A COMPARATIVE STUDY TO EVALUATE THE EFFICACY OF ORAL LACTOFERRIN FORTIFIED BOVINE COLOSTRUM WITH ORAL IRON IN THE TREATMENT OF IRON DEFICIENCY ANAEMIA

Dissertation Submitted To

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI

In partial fulfillment of the regulations

For the award of the degree of

M.D. (PHARMACOLOGY)

BRANCH VI

GOVT. KILPAUK MEDICAL COLLEGE AND HOSPITAL

CHENNAI

April 2015

CERTIFICATE

This is to certify that this dissertation titled **"A COMPARATIVE STUDY TO EVALUATE THE EFFICACY OF ORAL LACTOFERRIN FORTIFIED BOVINE COLOSTRUM WITH ORAL IRON IN THE TREATMENT OF IRON DEFICIENCY ANAEMIA"** is the bonafide original work done by **Dr. Taruni R.**, Post graduate in Pharmacology, under my overall supervision and guidance in the Department of Pharmacology, Govt. Kilpauk Medical College and Hospital, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Pharmacology (Branch VI).**

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DECLARATION

I solemnly declare that this dissertation titled "**A COMPARATIVE STUDY TO EVALUATE THE EFFICACY OF ORAL LACTOFERRIN FORTIFIED BOVINE COLOSTRUM WITH ORAL IRON IN THE TREATMENT OF IRON DEFICIENCY ANAEMIA",** is the bonafide work done by me at the Department of Pharmacology, Govt. Kilpauk Medical College and Hospital, Chennai, under the supervision of **Dr. RAMACHANDRA BHAT, M.D.,** Professor & H.O.D of Pharmacology, and guidance of **Dr. MALAR SIVRAMAN, M.D.,** Professor, Department of Pharmacology and **Dr. T. RAVINDRAN, M.D.,** Professor, Department of Internal Medicine, Govt. Kilpauk Medical College and Hospital, Chennai-600 010. This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of Degree of M.D. Pharmacology (Branch VI) examinations to be held in April 2015.

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ACKNOWLEDGEMENT

I would like to express my humble gratitude to **Dr.N.GUNASEKARAN, M.D., D.T.C.D,** Dean**,** Government Kilpauk Medical College and Hospital for giving me permission to carry out my dissertation work.

I would like to express my sincere gratitude to **Dr. Ramachandra Bhat**, **M.D.**, Professor and HOD, Department of Pharmacology, Govt. Kilpauk Medical College and Hospital, for introducing me to the world of medical research and riveting in me a strong foundation in ethics in medical research.

I am deeply grateful for the efficient support and guidance of **Dr. Malar Sivaraman, M.D.,** Professor, Department of Pharmacology, Govt. Kilpauk Medical College and Hospital, for her continued patience, commitment and dedication during the entire course of this endeavour.

I am also grateful to **Dr. T. Ravindran, M.D.,** Professor, Deprtamentof Internal Medicine, GOvt. Kilpauk Medical College and Hospital, for his enthusiasm and willingness to co-guide this thesis.

I extend my heartfelt gratitude to **Dr. T. Aruna, M.D.,** Professor, Department of Pharmacology, Govt. Kilpauk Medical College and Hospital who provided insightful inputs into the study and kept me focussed throughout the study period.

I also thank **Dr. J. Komathi, M.D., Dr. B.Sharmila, M.D., Dr. Jayaponmari, M.D.,** and **Dr. Sasikala, M.D.,** Assistant Professors, Department of Pharmacology, Govt. Kilapuk Medical College and Hospital, and my fellow postgraduate students for their help and their valuable support.

I would like to express my deep gratitude to **Dr. Komala, M.D.,** Assistant Professor, Department of Biochemistry, Govt. Kilpauk Medical College and Hospital**,** for administering the specialized lab tests with great care and clinical precision. The tireless work of performing the routine investigations by the team at the Biochemistry and Pathology laboratories were indeed of great help to me.

The enthusiasm and eagerness of the Principal of School of Nursing, **Mrs. Edwin Nirmala** percolated down to her students without whose cooperation this study would not have its cohort study population.

I am very thankful to the team from **M/s. Tablets (India) Ltd.** who keenly followed the vicissitudes of this postgraduate study and provided the Test Nutraceutical.

It would not be possible to put words to numbers if it weren't for the statistical inputs by **Ms. Valarmathi**.

This acknowledgement would be incomplete if I did not thank my family for their blessings and good wishes.

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LIST OF ABBREVIATIONS

ADR	-	Adverse Drug Reactions
ATP	-	Adenosine Triphosphate
BFU	-	Blast Forming Unit
BMP	-	Bone Morphogenic Protein
CHr	-	Haemoglobin Concentration in Reticulocytes
CKD	-	Chronic Kidney Disease
CNS	-	Central Nervous System
DC	-	Differential Count
Dcytb	-	Duodenal cytochrome b
DMT-1	-	Divalent Metal Transporter
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylene Diamine Tetra Acetate
ELISA	-	Enzyme Linked Immunosorbent Assay
ERC	-	Endosomal Recycling Compartment
ESR	-	Erythrocyte Sedimentation Rate
FDA	-	Food and Drug Administration
Fe/S	-	Iron/Sulphur
FE-Tf	-	Iron Bound to Transferrin
GABA	-	Gamma Amino Butyric Acid
GIT	-	Gastrointestinal Tract
GM-CSF	-	Granulocyte Monocyte Colony Stimulating Factor
HCP-1	-	Haeme Carrier Protein-1
HFE	-	Human hemochromatosis protein
HOx-1	-	Haeme Oxygenage-1
ICMR	-	Indian Council of Medical Research
IL	-	Interleukin

IQ	-	Intelligent Quotient
IRE	-	Iron Responsive Element
IRP	-	Iron Regulatory Protein
ITLN-1	-	Intelectin-1
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCV	-	Mean Corpuscular Volume
mRNA	-	Messenger RNA
NK	-	Natural Killer
PCBP-1	-	Poly R (c)-Binding-protein-1
PCV	-	Packed Cell Volume
PEP/LVET	-	Pre ejection period to left ventricular ejection time ratio
PPI	-	Proton Pump Inhibitors
R.P.M	-	Revolutions Per Minute
RBC	-	Red Blood Corpuscles
RDW	-	Red Cell Distribution Width
RNA	-	Ribonucleic Acid
SD	-	Standard Deviation
SGOT/PT	-	Serum Glutamic Oxaloacetic/Pyruvic transaminase
SLC	-	Solute Linked Carrier Protein
Steap3	-	Six Transmembrane Epithelial Antigen of Prostate 3
sTfR	-	Soluble Transferrin Receptor
TC	-	Total Count
TfR	-	Transferrin Receptor
TIBC	-	Total Iron Binding Capacity
TNF	-	Tumour Necrosis Factor
TSAT	-	Transferrin Saturation
UIBC	-	Unsaturated Iron Binding Capacity

WBC	-	White Blood Cells
WHO	-	World Health Organization

ABSTRACT

Title: A Comparative Study To Evaluate The Efficacy Of Oral Lactoferrin Fortified Bovine Colostrum With Oral Iron In The Treatment Of Iron Deficiency Anaemia

Introduction: Iron deficiency affects more than 2 billion people globally, with greater prevalence noted amongst women and children. Oral ferrous sulphate, the most commonly prescribed drug for treating this condition, is associated with 25 to 40% incidence of adverse drug reactions. This along with its variable bioavailability emphasise a need for better oral formulations. Lactoferrin, a glycoprotein structurally resembling transferrin, is believed to play a role in iron absorption. Hence a study was designed to evaluate the efficacy of oral lactoferrin fortified bovine colostrum in the treatment of iron deficiency anaemia.

Aim: To compare the efficacy of oral lactoferrin fortified bovine colostrum (as a single agent and in combination with ferrous sulphate) with oral ferrous sulphate in treating iron deficiency anaemia

Methodology: A prospective randomized open–labelled study was designed with 3 parallel arms and a study population of 68 anaemic women. The control arm was given oral ferrous sulphate 333 mg (containing 100 mg elemental iron) OD, the study arm was given lactoferrin fortified bovine colostrum 2g OD , while the combination arm received both. All treatment regimens were for 30 days. Baseline and post-therapy haemoglobin and iron parameters were assessed and analysed using student's paired t-test, ANOVA and Wilcoxon signed rank test.

Results: There was significant improvement in haemoglobin and iron parameters from baseline to post-therapy in the arms that received lactoferrin fortified bovine colostrum and was associated with fewer adverse events. The improvement in haemoglobin and iron parameters in the combination arm were comparable to the study arm. There were fewer adverse effects in the arms that received lactoferrin fortified bovine colostrum compared to ferrous sulphate arm.

Conclusion: Hence lactoferrin fortified bovine colostrum is a safe and efficacious treatment modality for iron deficiency anaemia and is associated with fewer adverse effects compared to oral ferrous sulphate.

Keywords: Iron Deficiency Anaemia, Lactoferrin fortified bovine colostrum, ferrous sulphate.

INTRODUCTION

Iron is an essential element required by the body for normal tissue oxygenation. Iron deficiency is the most common nutritional deficiency which affects more than 2 billion people worldwide.[1] In India, the prevalence of iron deficiency anaemia is as high as 74.3%, with women and children being affected more than the general population.[2] In Tamil Nadu alone, it affects 55.6% of women.[3]

Iron deficiency anaemia is associated with easy fatigability and decreased work performance in adults.[1] Pregnant women with iron deficiency anaemia have increased morbidities and mortality during their antenatal period with poor outcomes.[3] Children with iron deficiency anaemia manifest psychomotor developmental delays.[1] Hence it is essential to rectify the iron deficiency in an individual.

The current treatment modalities available such as oral ferrous sulphate and parenteral iron are fraught with many adverse drug reactions which lead to poor patient compliance and hence poor response to therapy.[4]

Our search for an effective, patient friendly treatment of iron deficiency anaemia led us to lactoferrin. Lactoferrin, a glycopeptide found in colostrum, belonging to transferrin family, is involved in absorption of iron from dietary sources.[5] Hence, supplementing lactoferrin should increase iron absorption. Its

efficacy in increasing iron parameters in pregnant women was proven in a few European studies.[77,78,83]

Lactoferrin fortified bovine colostrum (Laktrum) is a nutritional supplement available in India, with a high lactoferrin content. It is devoid of unpleasant side effects and therefore may improve patient compliance and result in better therapeutic outcomes. Hence a study was designed to assess the efficacy of lactoferrin fortified bovine colostrum in treating iron deficiency anaemia in Indian women.

REVIEW OF LITERATURE

IRON

Iron is a metallic mineral that constitutes most of Earth's Inner and outer core and is the fourth most abundant element on the Earth's crust. The magnetosphere generated by the spinning iron at the centre of the Earth is responsible for protecting the life force on earth from harmful solar winds and radiation.[6]

Three and a half billion years ago, life started on this planet in an aerobic, hyperthermic, hyperbaric environment with the transfer of electrons from mineral sources such as iron to electron acceptors.[6] This process gave rise to the building blocks of life in the form of various carbon containing organic compounds. Iron's crucial role in the creation of organic compounds is best summed up by R.J.P. William's prophetic remark, expressed in *Nature*, 1990, "Energy capture based on Fe/S compounds, now and perhaps before there was life, is as important as DNA in life's history."

Over the next 1.5 billion years, photosensitizing cyanobacteria led to the insidious addition of oxygen into the atmosphere which dramatically changed life on earth. The entry of oxygen revolutionized the planet's metabolic numbers from an anaerobic reaction that generated a mere 2 ATPs per molecule of glucose to 36 ATP per molecule of glucose. The presence of oxygen also led to a meandering course of iron metabolism as ferrous iron found itself being oxidized to insoluble ferric iron

This change in the form of iron availability had profound effects that is evident even now where all organisms struggle to acquire, transfer and store this precious metal, iron.

Iron Absorption and Metabolism in Lower Organisms

Iron, in the presence of oxygen, forms oxides which are insoluble and hence unavailable for use by living matter. Organisms have overcome this by way of various cellular mechanisms that facilitate the absorption of iron in a biologically useful form. Microbes secrete small, high-affinity iron chelating molecules known as siderophores which enable them to capture iron from their environment.[7] Yet, others like yeast have devised mechanisms to reduce iron from its insoluble ferric form to soluble ferrous form.[8]

Iron Absorption and Metabolism in Higher Organisms

The mechanisms for iron absorption and utilization in higher organisms parallels those found in lower organisms. Iron absorption and metabolism has evolved to a complex, tightly regulated mechanism in humans. As early as 1938, it was established by Mccance and Widowson that iron is not excreted by the body.[9] It was understood that iron homeostasis in man is unique as it is regulated by its absorption and not by its metabolism or excretion. It was only during the turn of the century, 60 years later, after the discovery of new players in the iron-absorption pathway that some clarity on this subject has emerged.

DIETARY IRON

Humans ingest approximately 12 to 18 mg/day of dietary iron, of which only 1 to 2 mg is absorbed. Adult men and non-menstruating women require 13 mg/kg/day of iron while menstruating women require 21 mg/kg/day. The iron requirement can go up to 80 mg/kg/day in the last two trimesters of pregnancy.[10]

Iron may be ingested in two forms, either 'Haeme' iron or 'Non-haeme' iron. Haeme iron is sourced from the consumption of myoglobin and haemoglobin found in meat, fish and poultry. It accounts for 10–15% of the total iron intake in meat-eating populations and is responsible for more than 40% of the iron absorbed by the body. Non-haeme iron is sourced in diets rich in plant based foods such as cereals, legumes, pulses, fruits and vegetables. Non-haeme iron may exist as food ferritin, iron minerals or iron complexes. Non-haeme iron requires acid digestion and is dependent on dietary enhancers and inhibitors while haeme iron is minimally affected by dietary factors. [11] [Table 1]

Table 1: Factors Modifying Dietary Iron Absorption

Type of Dietary Iron	Inhibitors	Enhancers
Haeme iron	None	None
Non-Haeme iron	Calcium Fibre Phytates, Tannins Polyphenols from Tea/Coffee/Wine Antacids	Ascorbate Citrate Amino Acids

Iron Absorption by Enterocytes

The extraction of iron from either haeme or non-haeme sources follow two convergent pathways as discussed below. Iron absorption occurs in the duodenum and upper jejunum. Two transport proteins, **DMT-1** (Divalent Metal Transporter) and **HCP-1** (Haeme carrier Protein) have been identified and implicated in the transport of iron to the cytosol of the enterocyte.

Absorption of Non-Haeme Iron by Enterocyte

Iron from non-haeme sources exist as ferric iron. For absorption of non-haeme iron, the ferric iron is first reduced to the ferrous form by **duodenal cytochrome b (dcytb)**. Dcytb is an ascorbate-dependant ferric reductase present in the duodenum. [12] The reduced ferrous iron then enters the duodenal enterocytes via the apical membrane with the help of **Divalent Metal Transporter-1 (DMT-1).** DMT-1 is a glycoprotein with 12 transmembrane domains with a broad range of divalent substrates such as Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+}.[13] DMT-1 is expressed by the intestinal cells concomitantly with dcytb and is upregulated in the presence of iron deficiency.[14] Hence the uptake of iron from non-haeme sources requires its reduction by dcytb followed by its transport into the enterocyte by DMT-1.[Figure 1 a]

Absorption of Haeme Iron by Enterocytes

Due to its hydrophobic nature, haeme was thought to passively diffuse into the enterocyte but this has been disproved by the discovery of **Haeme carrier protein-1 (HCP-1)**. HCP-1 (a.k.a SLC46A1) is expressed abundantly in the

duodenal enterocytes and transports iron within haeme from the apical membrane to the cytosol of the enterocyte. The expression of HCP-1 appears to be regulated by iron levels. The transport of haeme by HCP-1 is a saturable process.[15] Once inside the cell, haeme acts as a substrate for **Haeme Oxygenase-1**(HOx-1), an enzyme discovered by Tenhunen et al.[Figure 1b] Haeme Oxygenase-1 attacks the α-methylene bridge of the haeme macro cycle in an oxygen-dependent manner and causes the release of iron along with biliverdin and carbon monoxide.[6,16]

Export of Iron from Enterocyte

Iron in the enterocyte can either be stored as ferritin or exported to the blood to be carried to tissues by transferrin. The export of iron from the enterocyte is facilitated by an iron transporter known as **ferroportin**. Ferroportin (aka SLC40A1) was first identified by positional cloning of the causative gene causing hypochromic anaemia in 'Weissherbst' zebrafish.[17] Subsequently, ferroportin was found in the polarized basolateral membrane of duodenal enterocytes, basal cells of the placental syncytiotrophoblasts and the cytosol of cells of the reticuloendothelial system. Human ferroportin is encoded by chromosome 2q and consists of 571 amino acids with conserved hairpin loop sequences. It has been established as a major transporter, if not the sole transporter of iron and transports iron in the ferrous state.[18]

Transfer of Iron to Transferrin

As iron can only be transported in the ferric state by transferrin, ferroportin is often associated with proteins that serve as ferrioxidases. One such protein is **Hephaestin,** which is a membrane bound multi-copper oxidase analogous to ceruloplasmin. Hephaestin is expressed in the intestinal villi whereas ceruloplasmin is found in macrophages, liver, brain, lung and astrocytes.[17] The former is crucial in the initial stage of incorporation of iron in transferrin, while the latter takes over that function at a later stage. Animal studies in mice with aceruloplasminemia suggest a facilitatory role of ceruloplasmin in the binding of iron to transferrin.[10]

Iron Transport by Transferrin

Ferric iron in the plasma is scavenged by transferrin (Tf) and delivered to tissues that either utilize or store the iron. Transferrin, an 80Kda glycoprotein encoded by chromosome 3q21-25, is synthesized by hepatocytes and secreted into the plasma. Transferrin is composed of a single bilobed chain that contain N-(Amino) and C-(Carboxy) lobes, each of which have two domains, referred to as N1, N2, C1, and C2. Each transferrin molecule has two iron binding domains that are located in the clefts between the two lobes. The binding and release of iron occurs by conformational changes brought about by the twisting of N1, N2, CI and C2 resulting in the opening or closing of subdomains of each lobe.[19]

Iron-bound transferrin normally has only one-third of its sites occupied, which facilitates further sequestration of any potentially toxic iron in the plasma. Hence transferrin plays a protective role by reducing the generation of free radicals by sequestering free iron in the plasma.

Transferrin is responsible for the transport of both endogenous and exogenous iron, a dynamic function it performs over 10 times a day in order to sustain normal erythropoiesis. It is astounding that although transferrin accounts for only 0.1% of the body's iron (~3mg), it is responsible for the transport of 30 mg of iron that is used to synthesize haemoglobin for 200 billion RBCs.[6] Circulating iron-bound-transferrin is the only source of iron to most cells except mature RBCs, enterocytes and the brain.

Transport of Fe-Transferrin into Cells

Great care has to be exercised in the transport of transferrin bound iron into the cytosol of the cell. The cell has to extract the tightly bound iron from transferrin, all the while ensuring that no harmful free radicals are generated in the process. The unbound potentially toxic iron will then have to be delivered to the site of functional assimilation in the cell which is usually the mitochondrion.[6]

Transferrin Receptors

Iron-bound transferrin (Fe-Tf) binds to a highly specific membrane bound transferrin receptor (TfR) that serves as a gatekeeper, regulating the entry of iron into cells that either utilize or store it. Cells that have a requirement for iron, exhibit

transferrin receptors at their cell membrane. There are two types of transferrin receptors, TfR1 and TfR2. Both are polypeptides with 3 domains; an apical domain, a protease-like domain and a helical domain. TfR1 is found in all tissues other than mature RBCs. Its expression is regulated by cellular iron level via HFE (human hemochromatosis protein). TfR2 is found mainly in hepatocytes and duodenal cells. It has a lower affinity for Fe-Tf than TfR1 and is not regulated by cellular iron levels.

TfR1 knockout mice are embryologically lethal while its deficiency results in low tissue iron levels. Deficiency of TfR2 leads to the development of hemochromatosis which is a state of iron overload. TfR2 regulates the expression of hepcidin, which in turn is considered to be the master regulator in iron homeostasis.[17]

Fe-Tf-TfR Internalization

Receptor mediated uptake of Fe-Tf is a complex process involving targeting, signalling, docking and movement of the complex into the cell. It is initiated when 2 molecules of Fe-Tf bind to the arginine-glycine-aspartate sequence of the helical domain of the transferrin receptor.[9] The signal for internalization of the Fe-Tf-TfR complex is provided by the tyrosine moiety located in the N-terminal of the cytoplasmic domain of TfR.

A clathrin mediated endocytosis of Fe-Tf-TfR complex occurs, followed by fusion of the clathrin coated vesicle with an endosome which is referred to as the sorting endosome. The next fate of Fe-Transferrin-TfR complex has not been lucidly

chalked out. It has been observed to exist in a collection of tubular structures associated with microtubules known as the endosomal recycling compartment (ERC).[6]

Release of Iron from Fe-Transferrin-TfR Complex

The exact mechanism of release of iron from the Fe-Transferrin-TfR complex is hazy though it has been proposed that the acidic pH of endosomes is responsible for the dissociation of iron from the Fe-Transferrin-TfR complex. Iron free Transferrin-TfR exits the ERC and resurfaces back to the plasma membrane to ferry another load of iron.

Meanwhile, the iron in the endosome is reduced to its ferrous form by a ferric reductase. One such ferric reductase, STEAP3 has been found to perform this function in erythroid precursor cells.[6] The reduced iron is transported out to the cytosol by means of another divalent metal transport protein. The iron is sent to the mitochondria for utilization in metabolic processes or is stored as ferritin.

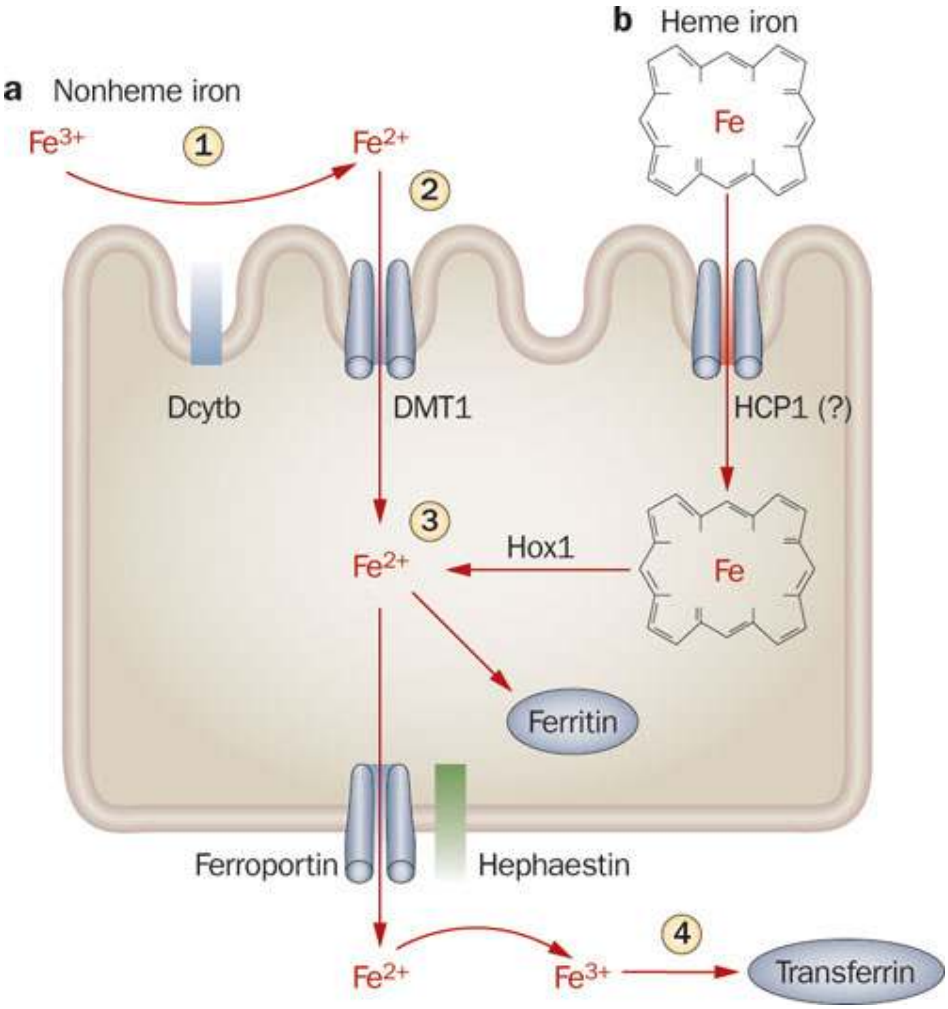


Figure 1: Absorption of Dietary Iron [87]

1. Reduction of non-haeme ferric iron to ferrous iron by Duodenal cytochrome b (Dcytb)
2. Transport of reduced iron into enterocyte by Divalent Metal Transporter (DMT-1)
3. Transport of iron by Haeme Carrier Protein (HCP-1). Release of iron from haeme iron by haemeoxygenase-1 (HOx-1)
4. Transport of iron from enterocyte to plasma ferritin by Ferroportin and Haephestin.

FERRITIN

The storage form of iron is ferritin, which is a ubiquitous spherical protein found in the mitochondria, cytosol, and nucleus of cells as well as in the serum. It is formed by the assembly of 24 subunits consisting of a varied ratio of H (heavy) and L (light) chains, both encoded by different genes. The subunits of ferritin assemble into a shell like structure with an 8nm central core capable of housing 4500 molecules of iron as ferric oxy-hydroxide phosphate. Ferritin may exist as tissue ferritin, serum ferritin or mitochondrial ferritin.

Tissue Ferritin

A specialized protein, Poly R (c)-Binding-protein-1(PCBP-1) chaperones iron from the cytosol to the core of ferritin. The L-subunit is responsible for the uptake of iron into the ferritin core. The ferroxidase activity of H-subunit is responsible for the oxidation of Fe^{2+} enabling mineralization of iron within the protein. The ratio of H to L varies from tissue to tissue. Heart cells and neurons exhibit a predominance of H subunit while L-subunits are predominantly expressed by the liver and spleen.[17]

Serum Ferritin

A glycosylated form of ferritin composed predominantly of L subunits can be found in the circulation. As the iron content of serum ferritin is low, it is not expected to play a major role in iron storage or transport. It serves as a highly specific bio-marker of tissue iron stores. The normal serum ferritin levels range from 15 to 150 microgram per litre. Serum ferritin levels help differentiate iron deficiency

anaemia from anaemia of chronic disease as in the latter case, ferritin stores will be normal. [17]

Mitochondrial Ferritin

Mitochondrial ferritin is synthesized in the cytosol as a precursor polypeptide that is later targeted into mitochondria by an N-terminal leader sequence. It does not play a major role in the utilization of iron by the mitochondria though its expression is significantly increased in patients with sideroblastic anaemia.[17]

Iron Utilization

Iron from ferritin can be mobilized as and when required and utilized only by its degradation. This may occur by two pathways, either a lysosomal or a proteosomal pathway. A deficit of iron must be there to trigger off either of the pathways. The lysosomal pathway leads to the complete lysis of ferritin followed by the release of its iron stores. For utilization of iron outside the storage cell, ferritin is transported with the help of ferroportin. This is particularly evident in macrophages. In the proteosomal pathway, the export of ferritin-derived-iron from the proteosome leads to monoubiquitination and degradation of the remnant apo-ferritin. In either case, the structured assembly of ferritin is destroyed in order to utilize the iron stores of ferritin. The ferritin-derived-iron is utilized for the running of the metabolic machinery of a cell rather than for erythropoiesis. Iron for erythropoiesis is derived directly from transferrin, the uptake of which is facilitated by the expression of soluble transferrin receptors on the bone marrow cells.[17]

Iron Regulation

The fluctuation of iron between ferrous and ferric states is a double edged sword, as the character of iron that facilitates electron transport in cellular respiration is also responsible for the generation of toxic labile ions. Most cytoplasmic iron exists in the ferrous state which on losing an electron forms toxic free radicals. Fenton reaction which occurs when ferrous iron interacts with H_2O_2 generates ferric ion, OH^- and hydroxyl radicals. These free radicals may result in lipid peroxidation and oxidative damage to macromolecules found in their vicinity. Hence cellular and plasma iron levels have to be tightly regulated.

The absorption of iron accounts for a mere fraction of the iron in the body with over 90% arising from the recycling of senescent RBCs. In an iron deficient state, the body increases its iron absorption by 3 to 5 folds. On the contrary, in an iron overloaded state, the body adopts the 'mucosal block phenomena' where iron bound to apo-ferritin in an enterocyte is shed off from the GIT along with the gastric epithelial cells. When there is excess of iron, ferroportin fails to transport absorbed iron out of the enterocyte.[6]

Much of the regulatory mechanisms are directed towards the release of stored iron, its transport and its recycling from cellular sources. Currently, the main factor regulating these processes is Hepcidin. It is secreted by the liver as a 25 amino acid peptide hormone. Hepcidin levels are decreased in hypoxia and anaemia and are increased in the presence of iron and inflammation. Hepcidin binds to ferroportin resulting in its internalization and degradation thereby preventing the entry of iron to plasma.[6] Conversely, decreased expression of hepcidin leads to increased surface

expression of ferroportin and thereby increased iron absorption. Hepcidin transcription, in turn, is regulated by the SMAD-4 mediated BMP (Bone Morphogenic Protein) receptor signalling pathway via other proteins such as HFE and Hemojuvelin.[10,20] Certain cytokines such as IL-6 are also believed to regulate its expression.[17]

Other proteins such as IRP-1, IRP-2 (Iron Response Proteins) bind to IRE (Iron Responsive Elements) in the untranslated regions of mRNA of regulatory proteins and are responsible for regulating the expression of key proteins in iron homeostasis.[6]

Role of IL-6 in Iron Regulation

IL-6 is believed to a play a role in iron homeostasis by both a hepcidin dependant and independent pathway. Increased levels of IL-6 result in an increased transcription of hepcidin by hepatocytes. This results in greater degradation of ferroportin resulting in less iron transfer from the enterocytes. Increased IL-6 can also directly cause the down-regulation of ferroportin mRNA expression resulting in decreased iron absorption.[21]

ROLE OF IRON IN THE BODY

The human body contains 3 to 4 g of iron, 60% of which is found circulating in the blood in the form of haemoglobin, 15% as myoglobin and the remaining 25% is stored as ferritin.[22] Iron, by virtue of being a constituent of various proteins and enzymes, plays an essential role in a wide spectrum of biological processes ranging from tissue oxygenation, energy metabolism to bactericidal actions of the immune system.

Role of Iron in Tissue Oxygenation

The importance of tissue oxygenation was stressed by J.B.S. Haldane who stated that, "Anoxia not only breaks the machine but also wrecks the machinery".

Tissue oxygenation is a process where oxygen from the lungs is transported to the cells of the tissues with the help of haemoglobin found in red blood cells. Iron in the ferrous state is capable of carrying oxygen, while iron in deoxygenated haemoglobin exists in the ferric state. Thus iron found in the porphyrin ring of haemoglobin is crucial for the iron carrying capacity of haemoglobin. Not only is iron important for the functioning of haemoglobin, but adequate iron levels are required for normal erythropoiesis. A study done in 1964 by Noyes et al., found that 90% of injected radio iron could be traced to haeme from the bone marrow within an hour. In iron deficiency, the haemoglobin content of RBC is reduced resulting in their microcytic hypochromicity.[6]

Role of Iron in Cellular Metabolism/ Respiration

The transfer of electrons to oxygen by ferrous iron and vice versa is the basis for the electron transport chain that generates ATP required for cellular functioning.[24] Iron is required by enzymes such as aconitase, succinate dehydrogenase, isocitrate dehydrogenase of the citric acid cycle**.**[6,23] **Hence cellular respiration would come to a standstill if it were not for iron.**

Iron is also an important constituent of various enzyme systems notably the cytochrome oxidases that mediate the metabolism of various endogenous and exogenous compounds.

Role of Iron in DNA Metabolism

The synthesis of DNA requires the conversion of ribonucleotides to deoxyribonucleotide by ribonucleotide reductase which requires iron for its optimum action. Iron serves as a co factor for xanthine oxidase which is responsible for the catabolism of purines. Hence the presence of iron is crucial for normal synthesis of DNA and its degradation.[23]

Role of Iron in CNS

In addition to its role in neuronal metabolism, iron is a constituent of an enzyme known as protoheamoxygenase. This enzyme is located in oligodendria and is responsible for cholesterol synthesis required for myelination in the CNS. Iron is also an important constituent of monoamine oxidase and a co factor for tryptophan

hydroxylase, enzymes which are crucial for the metabolism of neurotransmitters. Iron deficient individuals exhibit altered GABA metabolism and a downregulation of dopamine receptors.[23, 24]

Antimicrobial Actions of Iron

Just as higher organisms, lower organisms require iron for their functioning. Hence it is logical to assume that the lack of iron plays a protective role against invading organisms. Our body responds to invasion by microbes by regulating iron trafficking which results in anaemia of chronic disease. On the other hand, certain microbicidal enzymes such as catalase and myeloperoxidase that are released by neutrophils during an acute infection require iron for their optimum activity. Iron plays a regulatory role both in specific and non-specific immunity by enhancing T-cell activation.[23] Hence optimal iron levels are required for the antimicrobial actions of iron.

Other Roles of Iron in the Body

Iron plays versatile roles in various enzymatic reactions. Haeme synthase and uroporphyrinogen decarboxylase in porphyrin metabolism are under feedback control of iron. Iron is a constituent of pigment synthesizing enzymes such as phenylalanine hydroxylase and homogentistic oxidase. The metabolism of phenylalanine is closely intertwined with that of catecholamines and thyroxine, deviations of which can cause endocrinal changes.[23]

Hence it is evident that iron plays a multi-faceted role in tissue oxygenation and metabolism and inarguably its deficiency will have undesirable effects.

IRON DEFICIENCY ANAEMIA

Iron deficiency (sideropenia or hypoferrimia) is one of the most common nutritional disorders worldwide that affects over 2 billion people.[1] WHO estimates a prevalence of iron deficiency anaemia to be between 25% and 43% in developing countries.[25, 26][Figure 1.] In India, the prevalence is as high as 74.3%, with women and children being affected more than the general population.[2]

Iron deficiency is a condition where there is a dearth of mobilizable iron stores in the body. This occurs when the absorption of iron is insufficient to meet the demands of the body. An increase demand for iron is seen in people living in high altitudes, growing children and pregnant women. The deficiency of iron manifests as a wide spectrum of disease from the asymptomatic latent iron deficiency to the symptomatic iron deficiency anaemia. Mild to moderate forms of iron deficits show functional tissue impairment even in the absence of anaemia. The more severe stages of iron deficiency are associated with anaemia. Hence iron deficiency anaemia is a subset of iron deficiency.

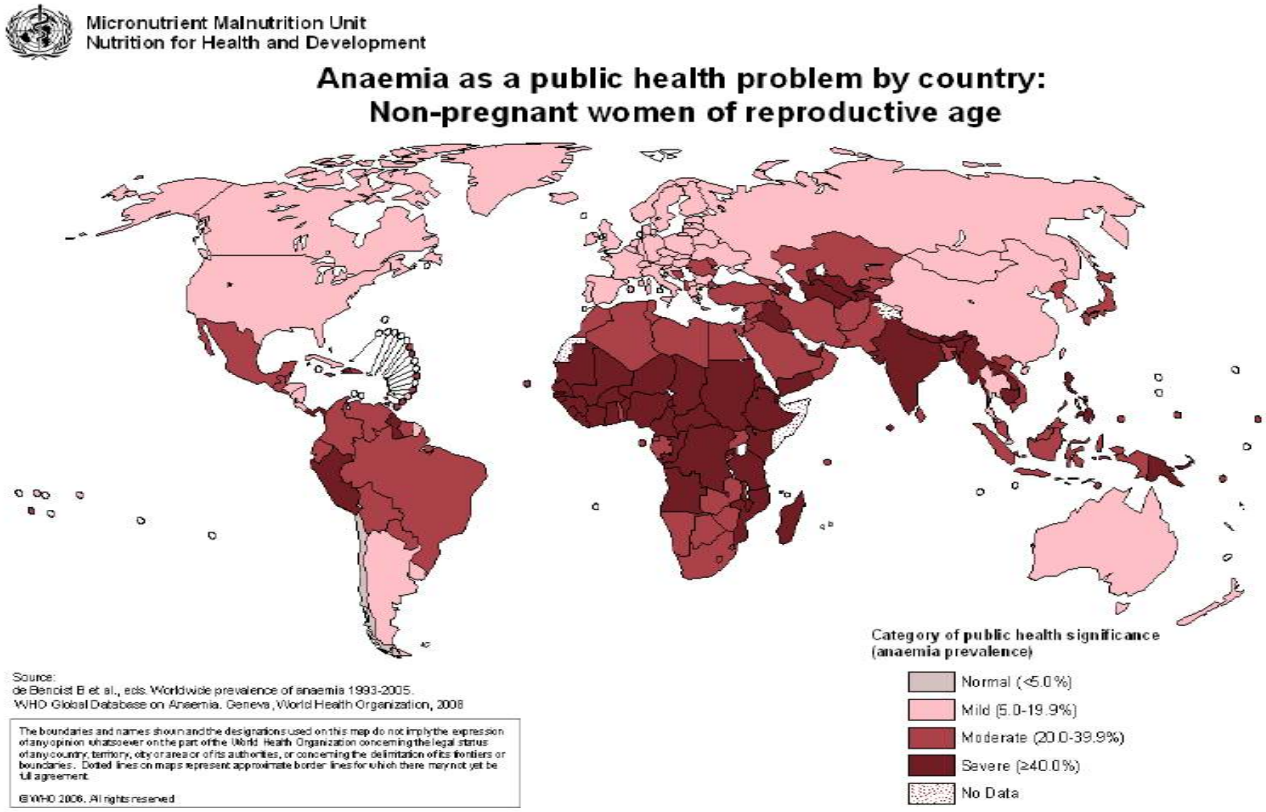


Figure 2: Global prevalence of Anaemia in not pregnant women [25]

DEFINITION OF ANAEMIA

An individual is said to be anaemic when his/her haemoglobin falls under 2 standard deviation of the mean haemoglobin of a population of same age, sex and altitude. WHO defines anaemia as a haemoglobin value less than 11 g/dl (at sea level). [4] ICMR has categorized the severity of iron deficiency based on haemoglobin levels as mild, moderate and severe.[27][Table 2]

Table 2: Categorization of Anaemia Based on Haemoglobin Levels

Severity	Haemoglobin Concentration (g/dl)
Mild	8-11
Moderate	5-8
Severe	<5

Such a categorization proves to be a useful tool in deciding appropriate treatment modalities and comprehensive evaluation of the condition.

The following pages discuss the aetiology, clinical features, diagnosis and current treatment available for iron deficiency anaemia.

AETIOLOGY OF IRON DEFICIENCY

In developing countries such as ours, a multitude of factors ranging from malnutrition, malabsorption, parasitic infestations, phytate rich diets, lack of motivation to seek medical help, early marriages, teenage pregnancies, or multiple consecutive pregnancies may be responsible for causing iron deficiency anaemia. An iron deficient state may be borne out of inadequate intake, insufficient absorption, increased utilization or excessive loss from the body.[1]

Certain food faddisms that are inherently low in iron content lead to decreased iron intake. Diets rich in iron such as dates, green leafy vegetables and meat should be encouraged.

A pregnant women may require up to 1000 mg of additional iron to sustain a healthy pregnancy.[27] An iron deficit may arise in growing children and pregnant women if the increase in iron demand is not met by an increased intake of iron. An increased iron demand is also observed following administration of erythropoiesis stimulating agents such as erythropoietin in patients with chronic kidney disease.

There could be an excess loss of iron from the body by way of insidious, traumatic bleeding or menorrhagia where iron is lost to the outside world along with haemoglobin in the blood. Any cause of acute or chronic blood loss may result in iron deficiency. Adults lose about 1 mg of iron per day that could rise up to 10 mg

per day in a normally menstruating woman.[1] Frequent blood donations could precipitate an iron deficient state as there is a loss of 500 mg of iron per donation. Therapeutic phlebotomy that is undertaken in polycythaemia coupled with increased erythropoiesis may also result in an iron deficient state.[1]Chronic haemoglobinuria due to paroxysmal nocturnal haemoglobinuria or hemolysis due to mechanical heart valve may lead to excessive iron loss as well.[28, 29]

A vitamin A deficiency interferes with normal metabolism of iron and may be responsible for impaired iron absorption.[31] Decreased iron absorption can arise due to concomitant intestinal malabsorption syndromes such as inflammatory bowel diseases, celiac sprue or Whipple's disease.[1] The absorption of iron from the small intestine is also affected following intestinal resective surgeries.[32]

Lastly, and one of the most often implicated, is a decreased absorption of iron by the body. This may occur due to inadequate intake per se or due to abnormalities in the iron absorption pathway. Though genetic reasons for defective absorption have been identified in animals, it is poorly defined in humans.[30]

CLINICAL FEATURES AND SYSTEMIC MANIFESTATIONS OF IRON DEFICIENCY ANAEMIA

The importance of iron in health is exemplified by the derangements that occur in its deficiency. Iron deficiency and its associated anaemia can cause decreased stamina, easy fatigability, impaired cognition, altered audio and visual perceptions, infertility, increased antenatal mortality and morbidity and delayed psychomotor developmental milestones.[1]

Due to its vital role in cellular respiration, iron deficiency can present as decreased stamina or easy fatigability. A study undertaken in peripheral health centres observed that in only 1 in 52 patients presenting with fatigue, is the cause of fatigue anaemia.[33] On the other hand, detection of pallor as a screening procedure to pick up anaemia has a positive likelihood ratio of 4.5.[1]

Pregnancy

India contributes to 44% of the global maternal mortality rate. Pregnancy poses a unique situation where the increased demand for iron by the growing foetus, coupled with the 50% rise in plasma volume, precipitates dilutional anaemia associated with iron deficiency. 40% of perinatal maternal deaths and an increased incidence of premature delivery are associated with iron deficient pregnancies.[43] Increased prenatal, perinatal infant and maternal mortalities and morbidities are observed in iron deficient women so much so that the chances of a favourable pregnancy outcome is reduced by 30 to 45%. Abnormal implantation or defective

embryogenesis of heart, lung and brain have been observed to be associated with iron deficiency.[10] In addition the infants born to such women have less than half the normal reserve of iron.[43]

Growth In Children

The incidence of febrile seizures and breath holding spells is increased in infants who have iron deficiency anaemia. The physical and cognitive development of children with iron deficiency anaemia is impaired.[10] The importance of iron in growth is evident from studies that show improved growth following iron supplementation in iron deficient children. The improvement depends on the age of development of iron deficiency, dietary factors and presence of concomitant diarrhoea. Children who develop impaired immunity due to iron deficiency anaemia are highly susceptible to infectious diseases.[37, 44]

CNS and Behaviour

Experiments with iron deficient animals show altered neurotransmitters and behaviour that stress the importance of iron in normal brain function.[34] This has been observed in iron deficient humans as well who exhibit impaired cognitive performance and delayed psychomotor milestones in infants and children. An iron deficient brain results in diminished attention span, poor academic performance, depression, altered sleep patterns and reduced mental alertness. Electrophysiological measurements undertaken in children and adults document a neurological malfunction associated with iron deficiency. A Costa Rican study demonstrated that infants with moderate anaemia achieved lower IQ scores and poorer cognitive

performance on entry into school than children who were not anaemic in their infancy.[35] A study published in 1992 by Balin et al showed that adolescent girls on supplemental iron demonstrated increased ability to concentrate in school.[38] Hence it is imperative that iron deficiency anaemia is prevented amongst infants and children to ensure normal cognitive development in their early formative years.

Cardiovascular System

The resting heart rate of an iron deficient individual is increased. The microcytic hypochromic anaemia associated with iron deficiency anaemia leads to reduced oxygen carrying capacity of the red blood cells which results in a hyperdynamic circulation provided by a tachycardic heart. This may precipitate heart failure in patients with impaired heart function as it overburdens the system. Reduced ventricular function is observed with a reduced pre ejection period to left ventricular ejection time ratio (PEP/LVET). This ratio is normalized within days of iron therapy even before a documented rise in haemoglobin. ST depression may be observed in a treadmill test which is reversible following adequate therapy.[23]

Gastrointestinal Tract

The cells of the gastrointestinal tract are continuously proliferating and differentiating. Iron, which is essential for DNA synthesis and cellular differentiation, when deficient will affect the entire length of the GI tract. At the oral end, a patient may present with angular stomatitis, cheliosis or glossitis. The postcricoid oesophageal web found in patients with Plummer Vinson syndrome is responsible for sideropenic dysphagia. Atrophic gastritis and malabsorption

syndromes are found concomitantly with iron deficiency. In a study conducted by Mehta et al, 28% of patients with iron deficiency showed a reversal of malabsorption of D-xylose accompanied by a significant rise in haemoglobin following iron therapy.[23] This suggests that iron deficiency can also cause malabsorption and not necessarily always vice versa.

The compensatory increased absorption of iron in iron deficient patients who exhibit abnormal eating behaviours of pica may prove to be toxic due to the concomitant increase in absorption of cadmium and lead by DMT-1. Iron deficient children are particularly susceptible to lead poisoning by ingestion of chipped paints or inhalation of automobile fumes due to this.[23]

Immunity

Iron deficient individuals show increased susceptibility and morbidity to infections. Increased incidences of furunculosis, candidiasis and upper respiratory tract infections have been noted. This may be attributed to the diminished antimicrobial power of leukocytes due to impaired myeloperoxidase and phagocytic actions. The failure of lymphocytic replication on mitogenic stimulation results in reduction in the number of cells responsible for cell mediated immunity. Children on dietary supplements of iron have been known to reduce susceptibility to infectious diseases.[23] As microorganisms thrive in an iron rich environment, the high prevalence of iron deficiency anaemia in a developing nation such as ours may in fact prove to be a blessing in disguise.[42]

Musculoskeletal System

Easy fatigability and reduced exercise tolerance are common symptoms of iron deficiency anaemia. Studies conducted amongst agriculture workers in many countries such as Indonesia, India and Sri Lanka have demonstrated a linear relationship between iron deficiency and work capacity.[40] A rapid return to normal has been documented through iron supplementation.[41]

Diminished iron stores in myocytes and the decreased myoglobin that is observed in iron deficient states result in exercise intolerance. This is particularly notable in fast acting group of muscles. A study conducted by Mann S.K. et al. in Punjab, amongst iron deficient non-anaemic females showed improved endurance levels and physical performance following iron supplementation.[37,41]

Endocrine System

Iron deficiency is associated with impaired synthesis and catabolism of thyroid hormones and catecholamines. This results in altered neurological and musculoskeletal functions with impaired temperature control. Iron deficient individuals have low tolerance to cold. [23]

Skin and Appendages

Koilonochyia or spoon shaped (concave) nails is a well-known manifestation of chronic iron deficiency though exposure to petroleum products, trauma, high altitude or genetic causes may also result in this deformity.[39] There have been reports of premature greying or loss of hair, alopecia, acne and folliculitis in patients with iron deficiency with or without associated anaemia.[23]

Iron Deficiency and Drug Metabolism

The iron containing cytochrome p450 system, which is responsible for drug metabolism, may be affected in iron deficiency. This could lead to altered pharmacokinetics and pharmacodynamics of drugs. An altered creatinine clearance that sometimes accompanies iron deficiency could also alter metabolism of xenobiotics. Hence, altered absorption, increased cardiac output, redistribution of drugs and altered renal function may affect drug biotransformation.[23]

DIFFERENTIAL DIAGNOSIS

The clinical symptomology of anaemia is observed in megaloblastic anaemia caused by deficiency of vitamin B-12 or folic acid. Nutritional deficiency of Vitamin A or Vitamin C may mimic iron deficiency anaemia. Hereditary defects in haemoglobin synthesis or haemolytic conditions such as Glucose-6-Phosphate dehydrogenase deficiency and thalassemia may present with microcytic hypochromic anaemia. Anaemia of chronic disease can be differentiated from iron deficiency anaemia by the presence of normal to increased ferritin stores. Lead

poisoning presents with microcytic hypochromic anaemia but can be differentiated from iron deficiency anaemia by its characteristic signs and symptoms. Sideroblastic anaemia which may arise due to genetic causes or as part of myelodysplastic syndromes presents with microcytic hypochromic ringed sideroblasts.[4]

DIAGNOSIS OF IRON DEFICIENCY ANAEMIA

A patient exhibiting signs and symptoms of anaemia must be evaluated further for the presence of iron deficiency anaemia. To establish a diagnosis of iron deficiency anaemia, the patient must show laboratory confirmed evidence for the presence of anaemia as well as decreased iron stores. The former may be established by assessment of haemoglobin, haematocrit, peripheral smear and red cell indices, while the latter may be confirmed by assessing the iron parameters.

Investigating the levels of bone marrow iron is considered the gold standard for diagnosing iron deficiency anaemia but this technique is invasive and extremely painful.[37] Less invasive blood tests that assess haematological and iron parameters are available to evaluate the iron status of an individual.

Laboratory Diagnosis of Anaemia

The laboratory diagnosis of anaemia may be made based on results of haemoglobin, red cell indices and a peripheral smear, tests that are routinely prescribed for their sensitivity and cost-effectiveness.

Haemoglobin

Haemoglobin, the iron containing oxygen transporter found in blood can be measured by colorimetry. Venous samples that have been mixed well with EDTA to prevent clotting should be used for this assay. Haemoglobin gets converted to a coloured protein known as 'cyanmethhaemoglobin' whose intensity is measured by a colorimeter. Haemoglobin levels depend on various factors such as age, sex, parity, nutritional status and altitude of living. Normal haemoglobin levels are 14 to 16 g/d in males and 12 to 14 g/dl in females.[26] A haemoglobin value less than 2 Standard deviations from an age and sex matched population mean at the same altitude is suggestive of anaemia.[26]

Haematocrit

Haematocrit or packed cell volume (PCV) is the volume of red blood cells found per litre of blood. The normal PCV of males in 40 to 54%, and females is 36 to 48%. Like haemoglobin, PCV tends to vary with plasma volume. Hence a high PCV will be noted in a dehydrated patient whilst a low PCV will be noted in an antenatal woman. Either capillary blood or venous blood with EDTA added to it may be used for this analysis. The sample has to undergo centrifugation and the packed cell height divided by the plasma level height expressed as a percentage gives the packed cell volume. [49]

Peripheral Smear

Peripheral smear examinations of unclotted blood stained with Wright's stain provides clues to detect abnormalities in RBCs, WBCs and platelets. The size, colour and number of RBCs may be discerned by this cost-effective assessment of peripheral smear. Iron deficiency anaemia presents with a microcytic hypochromic picture. Thalassemia, sideroblastic anaemia and lead poisoning may also present with a similar picture. [37]

Red Cell Indices

Red cell indices, first described by Wintrobe in 1929, are used to describe the size (mean corpuscular volume) and haemoglobin concentration within an RBC (Mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration). Red cell distribution width (RDW) or red cell morphological index is used to quantify the variation in sizes of the RBCs. The size wise distribution of the RBCs can be depicted by a histogram known as the Prince-Jones curve or the coefficient of variation may be expressed as a percentage. The normal RDW ranges from 11.5% to 14.5% and is expected to increase in iron deficiency anaemia. The change in RDW is the first haematological change noted in the peripheral smear of an iron deficiency individual, even before the appearance of microcytic hypochromic RBCs.[49]

Mean corpuscular volume (MCV) Mean corpuscular haemoglobin (MCH) Mean corpuscular haemoglobin concentration (MCHC) are all calculated from

haemoglobin, haematocrit (PCV) and RBC count. Table 3 presents the normal values and the expected change in iron deficiency anaemia.[49]

Table 3: Red Cell Indices

Parameters	Normal	Iron Deficiency Anaemia	Calculation
MCV	87 ± 7 fl	Decreased	PCV/RBC count
MCH	29 ± 2 pg/ cell	Decreased	Haemoglobin/RBC count
MCHC	34 ± 2 g/dl	Decreased	Haemoglobin/PCV

PCV = volume of packed cells per 1000 ml of blood; Haemoglobin = Haemoglobin in g per 1000 ml of blood; RBC count = RBC count in millions per ml of blood

40% of individuals with iron deficiency will demonstrate a normocytic normochromic picture on peripheral smear analysis.[36] MCHC is considered to be a highly sensitive indicator for iron deficiency anaemia, though its value indicates the iron status of the body during the entire lifespan of the RBC (~120 days).[49] To obtain a recent assessment of the availability of iron for incorporation into new RBCs, the haemoglobin concentration in reticulocytes [CHr] is assayed. The CHr test compares favourably with TSAT and serum ferritin in predicting response to intravenous iron. [47]

Reticulocyte Count

Anaemia is accompanied by chronic tissue hypoxia which results in reflex increase in erythropoietin release and erythropoiesis. This is evident by the presence of numerous reticulocytes in the peripheral smear. Reticulocytes are immature anucleate erythroid cells in the peripheral blood with remnant extra nuclear RNA

which makes them 8% larger and more convoluted than their mature counterparts. On examination of the peripheral smear using Wright's stain, reticulocytes will exhibit a bluish hue due to its residual RNA content. A reticulocyte count is done by examining a stained preparation of peripheral smear and expressing as a percentage the number of reticulocytes among 1000 erythrocytes. Flow cytometry is a relatively newer modality to obtain the absolute reticulocyte count. In conditions such as iron deficiency anaemia, reticulocyte count is expected to increase as the marrow responds to treatment.[48,49]

The above findings are not specific to iron deficiency anaemia, as numerous conditions such as thalassemia, sideroblastic anaemia, haemolytic anaemia and lead poisoning may present with a haematological profile similar to that found in iron deficiency.[1]

Laboratory Evidence of Iron Status of the Body

There are two ways of confirming that the microcytic hypochromic anaemia is due to iron deficiency. One is to give a therapeutic trial of oral iron therapy for 1 to 2 months and noting at least a 1g/dl rise in haemoglobin or a 3% increase in haematocrit. [37] A definitive diagnosis of iron deficiency anaemia based solely on haemoglobin, haematocrit and red cell indices cannot be arrived due to the following reasons:

1. Due to the surrogate nature of the red cell parameters, the actual body iron status cannot be discerned.

2. A change in haemoglobin and haematocrit levels occurs only in the last few stages of iron deficiency and up to 40% of patients with iron deficiency may not show an alteration in their peripheral smear
3. The only way to confirm an iron deficient state in an individual utilizing haematological parameters is by noting a change in their values following therapy. Such a therapeutic trial is not justified in patients who do not have iron deficiency anaemia.
4. The haematological profile in iron deficiency anaemia may be present in other diseases as listed under the differential diagnosis.

Hence, in order to truly ascertain the iron status of the body, assessment of iron parameters must be made. The iron parameters include serum iron concentration, serum ferritin, transferrin saturation, total iron binding capacity, unsaturated iron binding capacity, soluble transferrin receptor and zinc protoporphyrin. The latter two tests have been in the experimental stage for the past two decades and are considered to reflect the iron stores of the bone marrow.

Serum Iron Concentration

As iron has the potential to induce free radical damage by Fenton's reaction, it is always found bound to transferrin in plasma. Less than 1% of serum iron exists in an unbound form.[6]

The normal serum iron concentration ranges from 65 to 165mg/dl. [55] The majority of iron is derived from the catabolism of senescent RBCs which is a dynamic process. The turnover time for iron bound to transferrin is high as iron is

constantly being shuttled from storage or absorptive sites to sites utilizing it. As plasma iron circulates only for 40 to 50 min, intra individual variations in serum iron concentrations of up to 15% are observed. A diurnal variation of 10 to 20% may also be noted as serum iron concentration decreases in late afternoon and evening.[49,50] Serum iron concentration is expected to increase following dietary absorption and decrease as a response to various interleukins during inflammation, infection or in case of anaemia of chronic disease. Hence, defining an iron deficit based solely on serum iron concentration levels may result in false positive and false negatives without providing information on the iron stores of the body.[36]

Serum Ferritin

Although ferritin is a storage form of iron and is found mostly in tissues and macrophages, some ferritin escapes into plasma and its plasma level is found to have a direct correlation to the iron stores. Serum ferritin concentration is a measure of the iron stores of the body. Serum ferritin concentration can be accurately measured using chemiluminescence or ELISA.

The normal ferritin level is 40-160mcg/l.[55] Day to day variations and intra individual variations are not as pronounced as for iron. Previously, iron deficiency anaemia was defined as serum ferritin concentration of < 12mcg/l in females and <15 mcg/l in males. However this was found to possess an inadequate sensitivity of only 25%. Hence the cut-off limit was raised to 30mcg/l for both males and females increasing the sensitivity to 92% and the specificity to 98%.[4] Due to its high sensitivity and specificity, this test has gained wide acceptance as a diagnostic test for iron deficiency anaemia. [37,51]

Serum ferritin concentration can be utilized to differentiate iron deficiency anaemia from anaemia of chronic disease with the former showing reduced iron stores and the latter showing normal to increased iron stores. Apo ferritin is an acute phase reactant which may be elevated in certain infections and inflammatory conditions resulting in a falsely elevated ferritin level. However, after careful elimination of co-existing infection or inflammation, serum ferritin level is a reliable test to define the iron status of an individual, especially in developing countries.[37]

Total Iron Binding Capacity (TIBC)

Transferrin, the iron transporter in plasma, is usually saturated to one-third its capacity. TIBC is a reliable method of discerning transferrin concentration where the total number of transferrin binding sites per unit volume of plasma is assayed.[47]

TIBC can be calculated as the sum of serum iron concentration and unsaturated iron binding capacity or assayed using end-point colorimetric analysis. In the latter method, excess iron is added to the sample to saturate transferrin followed by precipitation of unbound iron. The quantity of iron bound to transferrin is assayed to obtain its total iron binding capacity. Normal values range from 250 to 370 mcg/dl.[36] It is a stable indicator of iron status as it does not change until iron stores are depleted.

Increased TIBC values are noted in iron deficiency anaemia, acute liver damage, progesterone birth control pills and in late pregnancy, while decreased TIBC is observed in hemochromatosis, hemosiderosis, hyperthyroidism, nephrotic syndrome, anaemia of chronic disease and thalassemia.

Unsaturated Iron Binding Capacity (UIBC)

This is the fraction of iron that remains unbound to transferrin when excess iron is added to the plasma. It can be assayed using end point colorimetric analysis where excess iron added to the sample binds to transferrin and the unbound iron is made to react with a colouring reagent, the colour intensity of which will give the fraction of unbound iron. It can also be calculated from TIBC values and serum iron concentration as depicted in the formula below:

UIBC(mcg/dl)= TIBC(mcg/dl)-Sr.Iron(mcg/dl)

Normal values range from 155 to 355 mcg/dl. As the value depends on the plasma iron levels, day to day variation, diurnal variation within the same individual can be noted. An iron deficient individual will exhibit increased unsaturated iron binding capacity. [49,52]

Transferrin Saturation (TSAT)

This is a calculated value obtained by dividing serum iron concentration by TIBC and expressing the value as a percentage.

$$\text{TSAT \%} = \frac{\text{Serum iron concentration}}{\text{TIBC}} \times 100$$

Normal TSAT values range from 20 to 50%. In Iron deficiency anaemia, TSAT values lie less than 20%. TSAT <15% is considered to be insufficient to meet the requirements for normal erythropoiesis.[47, 55] TSAT values greater than normal indicate iron overloaded diseases such as hemochromatosis. As TSAT is directly proportional to serum iron concentration, any variation in serum iron concentration will directly affect TSAT values. Hence one can expect to note diurnal and day to day intra individual variations in TSAT values.

Assessing Iron Stores of the Bone Marrow

The absence of stainable iron in the bone marrow is considered to be the gold standard test in diagnosing iron deficiency anaemia. This test has the demerits of being a painfully invasive procedure and is also subject to subjective inferences. Hence a search for less invasive, objective tests led to the assay of soluble transferrin receptor and the zinc protoporphyrin/ haeme ratio analysis. The former value does not vary in anaemia of chronic disease and neither values are affected by the presence of inflammation.

Soluble Transferrin Receptor Assay

In iron deficiency, the erythropoietic cells of the bone marrow exhibit an upregulation of transferrin receptors, some of which get detached and are detectable in the circulation. An increased sTfR is not specific to iron deficiency as it is noted in patients with increased erythroblastic activity and in patients on erythropoiesis stimulating agents (ESA). The sensitivity for this diagnostic measures lies at 70 to 81% with a specificity of 59 to 71%. [47] The sTfR/Ferritin Index which is a ratio of

sTfR to log ferritin is found to possess increased specificity and sensitivity in diagnosing iron deficiency. There is little consensus on performing this test as more studies are required to assess its utility.

Zinc Protoporphyrin Assay

The terminal step in haeme synthesis pathway is iron chelation by protoporphyrin catalysed by ferrochelatase. Both iron and zinc compete for the metal binding site on ferrochelatase. When iron levels are decreased, zinc binds to the ferrochelatase and gets incorporated into the haeme moiety. It was conceptualized in 1966, that zinc protoporphyrin could be studied to evaluate the iron stores of the body. As zinc protoporphyrin is fluorescent, its presence even in low concentrations can be detected increasing the sensitivity of the test. This test serves as an indicator for the availability of iron for erythropoiesis. Zinc protoporphyrin to haeme ratio analysis is found to possess increased specificity and sensitivity in diagnosing iron deficiency anaemia.[53]

NEWER DIAGNOSTIC METHODS UNDER RESEARCH

Tests such as reticulocyte haemoglobin, percentage hypochromic erythrocytes, hepcidin levels and non-transferrin bound iron (NTBI) are under evaluation as potential tests for assessment of iron status of the body. [36]

CURRENT MANAGEMENT OF IRON DEFICIENCY ANAEMIA

Once diagnosed, iron deficiency anaemia can be treated by exogenous iron supplementation. Iron levels can be restored by dietary supplements, administration of oral, intravenous or intramuscular iron or by blood transfusion in severe cases. However, **the cause for iron deficiency has to be ascertained and treated** as a mere replenishment of the iron stores may not suffice for long. An intrinsically hypoplastic marrow would fail to recover following iron supplementation and likewise the responsiveness of the marrow may be masked by continued blood loss. Response to therapy would depend on the severity of the iron deficiency, mode of treatment, patient compliance and comorbidities that may influence the pharmacokinetics and pharmacodynamics of the therapy chosen. In most instances, where the cause of iron deficiency anaemia is nutritional, therapeutic interventions prove beneficial to the patient. [55]

Oral Iron

Ferrous sulphate taken orally is the treatment of choice in iron deficiency. The bioavailability of ferrous salts is thrice that of ferric salts. Ferrous salts are available as sulphate, gluconate, fumerate, succinate, aspartate or a polysaccharide-iron complex. The effective dose of the ferrous salt would depend on its inherent iron content, though variability in bioavailability due to the nature of the salt has not been noted.[56]

Ferrous sulphate is commercially available as a heptahydrated salt (FeSO4 • 7H2O) and contains 20% iron. Ferrous fumerate is water soluble, stable and tasteless with an iron content of 35% while ferrous gluconate has an iron content of 12%.[55]

The recommended dose to treat iron deficiency anaemia is 2 to 3 mg/kg/day given in 3 divided doses for adults. Pregnant women who require additional iron to support their growing foetus would need 15 to 30 mg/day of iron. **When the circumstances do not demand haste, 100 mg of elemental iron per day would be sufficient to treat the iron deficiency.** [55]

Concurrent administration of iron with antacids reduces the bioavailability of iron. Attempts of enhancing the oral bioavailability of ferrous salts by surface acting agents, carbohydrates, inorganic salts, amino acids or ascorbate have resulted in a significant increase in side effects. The bioavailability of oral ferrous salts is increased by one-third to half when taken on an empty stomach but is associated with an increased incidence of upper gastrointestinal side effects. However, certain delayed release preparations exhibit greater efficacy compared to ferrous sulphate when taken along with meals. It is also advisable not to use preparations that have been combined with folic acid, vitamin B-12 or cobalt in order to avoid difficulties in interpreting patient's response.

The efficacy of therapy measured by its hematopoietic effects depends on the initial severity of the deficiency. A patient with haemoglobin of 5g/dl would require about 2 months to achieve a rise to normal levels, while a patient with an initial haemoglobin of 10g/dl would only require half that time to achieve the same. Hence the duration of therapy depends on the baseline haematological and iron parameters.

The restoration of iron stores of the body would require many months of therapy. The rate of increase in iron stores decreases after 3-4 months of therapy. Patients with concomitant bleeds or inadequate diet would require prolonged treatment.[55]

The biggest drawback to oral iron therapy besides its variable bioavailability, is the presence of GI adverse effects such as nausea, diarrhoea, constipation, heartburn and epigastric discomfort.[57] At high doses, nausea and epigastric discomfort are more prevalent. This intolerance to oral iron is due to the amount of soluble iron in the upper GI tract and may also be due to psychological factors. The change in bowel habits is not that noticeable at higher doses and are probably due to the effect of iron on intestinal bacteria. 25% - 40 % of those treated with oral iron exhibit the above symptoms. The high incidence of adverse effects, poor adherence to the treatment regimen and variable bioavailability result in inadequate response to therapy.[55]

If no improvement in the iron status is noted after 3 to 4 months of therapy, a switch to alternative treatment modalities such as parenteral iron therapy is warranted.[55]

Parenteral Iron Therapy

Parenteral iron administration is the preferred route of therapy in patients who have failed to respond to oral iron. Common indications include patients who have undergone gastrectomy, gastrojejunostomy and other surgeries of the small intestine. It is also indicated in patients exhibiting severe intolerance to oral therapy and routinely prescribed for patients on erythropoietin.

Parenteral iron formulations consist of an iron core encapsulated by a stabilizing carbohydrate shell which ensures release of iron within the cells of the reticuloendothelial system with minimal side effects. The current parenteral iron formulations available are iron dextran, sodium ferric gluconate, iron sucrose and ferumoxytol.[58,59]

Iron Dextran

Iron dextran is a colloidal solution of ferric oxyhydroxide complexed with polymerized dextran and contains 50 mg/ml of colloidal iron. The use of low molecular weight dextran is preferred over high molecular weight dextran due to lesser toxicity associated with the former. Iron dextran is indicated in any patient with documented iron deficiency not responding to oral iron. It may be administered as a total dose infusion, injected intravenously or intramuscularly. Both intramuscular and intravenous routes of administration must be preceded by a test dose of 0.5 ml. When total dose infusion is administered in a weekly spaced manner, test doses should precede each dose. The patient should be observed for one hour following the test dose for signs of immediate anaphylaxis, vascular instability, respiratory distress, hypotension, tachycardia, chest pain and back pain, any presence of which precludes further treatment with iron dextran. Fever, malaise, lymphadenopathy, arthralgia and urticarial reactions can occur days to weeks following therapy with iron dextran in patients with concomitant connective tissue disorders such as rheumatoid arthritis.[55]

When a dose of less than 500 mg is given intravenously, iron dextran complex has a t-half of 6 hours. At a dose of more than 1 g, the reticuloendothelial

clearance of 10 to 20 mg/hour results in brownish discoloration of the plasma with elevated levels of serum iron for a period of 1 to 2 weeks. Intramuscular iron dextran is to be administered as a dose of 0.5 to 2 ml using Z-technique over the upper quadrant of the gluteal region. There are concerns of local reactions at injection site and possible malignant changes associated with the intramuscular route. The intramuscular route is less reliable than the intravenous route, as in the former, iron has to be mobilized by the reticuloendothelial cells of the lymphatics before being released from the dextran complex.[58]

Sodium Ferric Gluconate

Sodium ferric gluconate has a molecular size of ~29,500 Da and is administered at a dose of 62.5 to 125 mg intravenously. Unlike iron dextran, iron from ferric gluconate is directly delivered to transferrin within 24 hours. Sodium ferric gluconate has a lower incidence of anaphylaxis compared to iron dextran and is the preferred form of parenteral iron therapy. In patients on dialysis, sodium ferric gluconate ensures a transferrin saturation of 100%.[58]

Iron Sucrose

Iron sucrose is a complex of polynuclear ferric hydroxide in sucrose that is pharmacokinetically similar to iron dextran. Its adverse effect profile is similar to ferric gluconate although one study has warned of additional potential renal injury in the form of tubulointerstitial damage following repeated use.

Iron sucrose and ferric gluconate have been FDA approved for patients with Chronic Kidney Disease (CKD) though wider applications are being sought. These

second generation iron formulations have to be administered at a lower dose to prevent dose related hypotension. They are associated with a lower incidence of adverse effects in comparison to the first generation iron dextran. [59]

Ferumoxytol

Ferumoxytol is a newer intravenous semisynthetic preparation. It is a super magnetic iron oxide nanoparticle coated with carbohydrate. It is administered as fixed dose combinations in the treatment of anaemia due to chronic kidney disease. It can be given over 5 minutes and supplies 510 mg of iron per infusion. Rat paw oedema tests have demonstrated its low immunogenicity. It may, however, be associated with transient hypotension.[59]

Blood Transfusion

Blood transfusion is reserved for seriously ill patients who need a rapid correction of their iron status. Though there are guidelines indicating cut-off haemoglobin levels for blood transfusion in severe anaemia, clinical factors and availability of blood also have to be considered. Transfusion of packed cell is preferred over whole blood in order to avoid volume overload. [1]

NEED FOR ALTERNATIVE TREATMENT FOR IRON DEFICIENCY

Both oral and parenteral iron are fraught with accompanying adverse drug reactions. While the oral route presents with mild tolerable side effects in a greater percentage of users (up to 40%), fatal anaphylaxis has been noted with the parenteral route in up to 0.7% of the users. Parenteral iron therapy is associated with adverse drug reactions such as bradycardia, chest pain, hypotension or hypertension, nausea, vomiting, diarrhoea, abdominal pain, headache, fever, allergic reactions, pruritus, malaise, arthralgias, myalgias, and back pain.[58] 35% of patients receiving iron sucrose exhibit mild adverse effects such as headache, nausea and diarrhoea, while iron dextran users complain of phlebitis when concentrated solutions are given over a prolonged period of time. Due to the high risk of anaphylaxis in parenteral iron therapy or transfusion reactions following blood transfusion, oral iron is the treatment of choice for mild to moderate anaemia. However, even this modality of treatment is not very patient friendly due to increased incidence of mild tolerable adverse drug reactions and poor oral bioavailability. Hence a need for an alternative treatment formulation exists. One such alternative form of therapy is lactoferrin, an iron binding glycoprotein.

LACTOFERRIN

Lactoferrin is an 80KDa glycoprotein belonging to the transferrin group of proteins. It was discovered in 1939 by Sorensen and Sorensen who first isolated it as 'red protein' from bovine milk. In 1960, it was found to be the main iron binding protein in human milk by three independent laboratories (Groves, 1960; Johanson, 1960; Montreuil et al., 1960). Lactoferrin was found to exhibit 60% homology to transferrin. As this iron containing protein was extracted from milk and showed remarkable similarity to transferrin, it was named lactotransferrin and later renamed as lactoferrin.[5]

Lactoferrin has been subsequently found in the secretions of exocrine glands and in the granules of neutrophils. Over the years, since its discovery, it has been isolated, purified, and its chemical characteristics and structure been elucidated by various laboratories. The immunological, antibacterial, antiviral, and anti-parasitic activities of lactoferrin and its role in cell proliferation and differentiation have been extensively studied.

However, there is a dearth of studies defining its role in iron metabolism, especially in humans. Three European studies have shed some light on this subject and have suggested a plausible role for lactoferrin in the treatment of iron deficiency anaemia.[77,78,83]

Structure of Lactoferrin

The molecular structure of lactoferrin was elucidated in 1984. Lactoferrin consists of a single polypeptide chain of 703 amino acids, whose C – (carboxy) and N – (amino) terminal regions have been folded into two globular lobes that are connected by α-helix. Each lobe consists of two domains known as C1, C2, N1, and N2. The domains create an iron binding site on each lobe. [60]

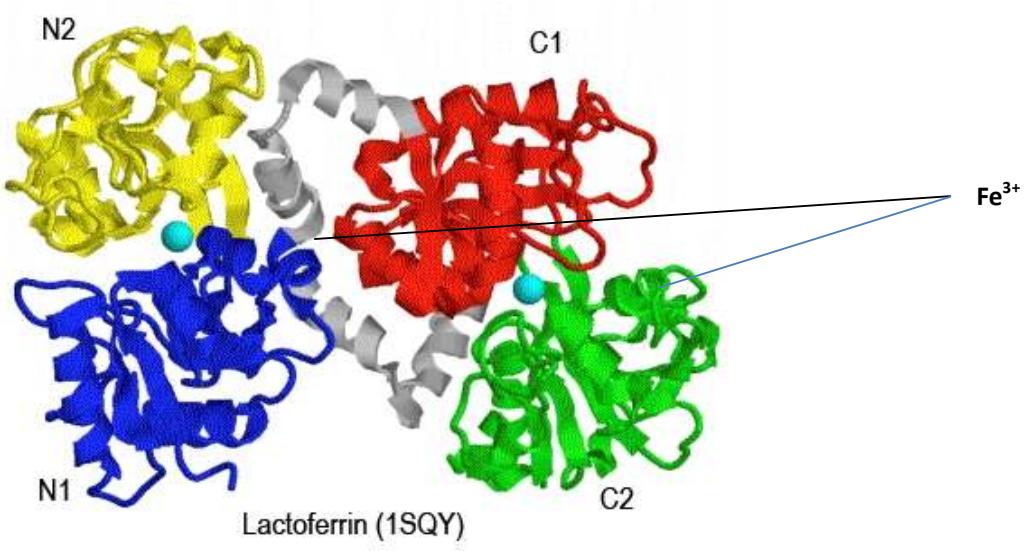


Figure 3: Richardson Ribbon Diagram of Lactoferrin. [61]

Isoforms of Lactoferrin

There are three different isoforms of lactoferrin that have been isolated -- lactoferrin-α, lactoferrin-β and lactoferrin-γ. Only lactoferrin-α is capable of binding iron. Lactoferrin-β and lactoferrin-γ demonstrate ribonuclease activity which may play a role in its antimicrobial action.[5]

Compared to transferrin, lactoferrin exhibits double the ability to bind iron. Each lactoferrin can bind two ferric ions. Based on the level of saturation in lactoferrin, it may exist in three forms. Apolactoferrin is its iron free form. The monoferric form carries one ferric ion while hololactoferrin binds two Fe^{3+} ions. The tertiary structure of iron-free lactoferrin is different from the iron laden forms. More recent studies suggest that lactoferrin fluctuates between the apolactoferritin form and the hololactoferrin form, but prevails in the former. [5] Lactoferrin, unlike transferrin, is a highly basic protein, which may be responsible for the preservation of its iron binding properties even in the acidic milieu of inflammatory loci.

Iron Binding Properties of Lactoferrin

The iron-lactoferrin bond is always associated with the concurrent binding of carbonate ion to the arginine residue of lactoferrin.[Fig.3] The histidine, tyrosine and aspartic acid residues of lactoferrin are important for its iron binding properties.[5]

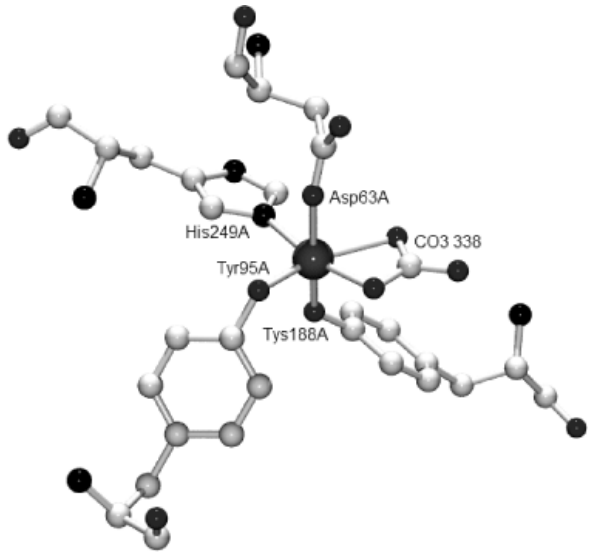


Figure 4: Iron Binding Sites of Lactoferrin [61]

Lactoferrin shows remarkable resistance to proteolytic degradation by trypsin and trypsin-like enzymes, the level of resistance being proportional to the degree of iron saturation. A low saturation of 10% may be responsible for its resistance to degradation even at a low pH of 4. Glycosylation at different sites on the molecule confers additional resistance to degradation. The most common glycosylating saccharide is mannose. Hexoses account for 3% of the glycosylation while hexosamines account for 1%.[5]

Lactoferrin is also capable of binding other ions such as Al^{3+}, Ga^{3+}, Mn^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+}, oxalates and carboxylates albeit with a lower affinity. In addition to ions, lactoferrin can also bind to lipopolysaccharides, heparin, glycosaminoglycans and DNA.[5]

Sources of Lactoferrin

Lactoferrin can be detected at the 2 cell stage of an embryo up until implantation. It is undetectable from then on till halfway through gestation. It is later detected in the neutrophil and epithelia of foetal reproductive and digestive systems.[5]

In adults, neutrophils are an important source of lactoferrin, where it is found mostly in the secondary granules. Due to its immunological functions, lactoferrin is found in most mucosal secretions such as uterine fluid, vaginal secretion, seminal fluid, saliva, bile, pancreatic juice, small intestinal secretions, nasal secretions, and tears.[63] It is also expressed and secreted throughout the collecting tubules of the kidney though its level in urine is quite low.[5]

The blood or plasma concentration of lactoferrin is negligible (0.02 μg/ml to 1.52 μg/ml) and varies based on age, gender, gestational age, stage of menstrual cycle, presence of infection, inflammation, tumour growth and extent of iron intake though the existing data pooled from different studies are incongruent.[5,64]

The highest concentration of lactoferrin is found in colostrum (7g/dl) followed by milk (1g/dl) where it is believed to be a major source of iron for infants.[63] Lactoferrin is purified and isolated from human or cow's milk though in the recent years recombinant lactoferrin is available. Human milk contains more lactoferrin but iron extraction from bovine lactoferrin is higher.[5]

Regulation of Lactoferrin Synthesis

The regulation of lactoferrin synthesis depends on the type of cell producing it. Lactoferrin synthesized by the mammary glands is controlled by prolactin. Its production in reproductive tissues is controlled by oestrogens and epidermal growth factor. [5] The hormonal effects on lactoferrin concentration is further evident by its increased concentration during the proliferative stage of the menstrual cycle. Its concentration also changes during pregnancy with a progressive rise up to the 29th week after which it plateaus. This rise is attributed to the increased synthesis by the endometrium and the mammary glands as well as to the leucocytosis that accompanies pregnancy with increased concentration in the neutrophils.[5]

In neutrophils, the production of lactoferrin depends on the stage of maturation with a freeze on synthesis in mature neutrophils. It is synthesized during the differentiation of promyelocyte to myelocyte and is stored for later use in secondary granules. Exocrine glands continuously produce and secrete lactoferrin.

Neutrophil-derived lactoferrin differs from lactoferrin derived from milk, in lacking the terminal fucose residues in its glycan chains.[63]

Lactoferrin Receptors

Lactoferrin receptors, typical for each cell type, have been found on the surface of hepatocytes, mucosal epithelial cells, fibroblasts and leucocytes including macrophages. Specific lactoferrin-binding proteins have been found in the intestinal microvilli of rabbit, mouse, and rhesus monkey suggesting a role for lactoferrin in iron absorption. These proteins vary in their molecular weight and their binding characteristics.

The receptor for lactoferrin found in the intestine is known as intelectin-1 (ITLN-1). It is a calcium dependent galactofranose specific lectin (38 Kilo Daltons) encoded by the ITLN-1 gene. ITLN-1 exists as a soluble form or is found bound to the membrane of small intestinal brush border cells. The latter form of ITLN-1 is primarily responsible for mediating lactoferrin uptake by enterocytes. It is inserted through a glycosylphosphatidylinositol anchor or attached to glycolipid enriched lipid raft microdomains through its lectin activity.[65] A Japanese study conducted by Kouichirou Shin et al. in 2008, demonstrated the ability of human ITLN-1 to bind bovine lactoferrin suggesting the lack of species specificity for the receptor.[66] 90% of the lactoferrin that is bound to its specific receptor on enterocytes, is degraded with the release of ferric ions. Decreased intracellular iron may result in increased expression of ITLN-1 facilitating an increased absorption of lactoferrin bound iron.[65] The mechanism of how ITLN-1 mediates iron uptake from lactoferrin is still unknown. [Figure 5.]

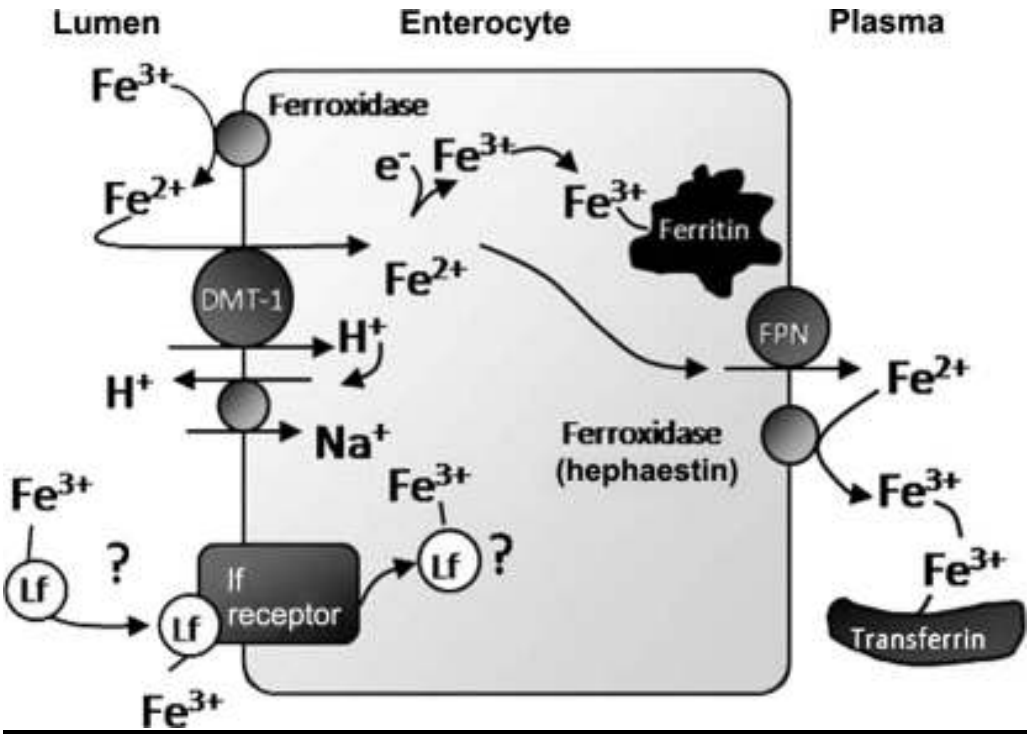


Figure 5 :Uptake of Iron-lactoferrin[45]

More than 60% of lactoferrin has been demonstrated to be absorbed by the adult human intestine. A study conducted in 2001 by Freddy J. Troost et al. in Netherlands demonstrated that lactoferrin is resistant to degradation by gastric juices.[46] Lactoferrin is metabolized and eliminated by two main mechanisms; one involving receptor mediated endocytosis by phagocytic cells, the other being a direct uptake by the liver. The phagocytic cells such as macrophages and monocytes subsequently transfer the iron content of lactoferrin to ferritin. The kidneys may play a role in the elimination of lactoferrin as traces of lactoferrin and its fragments of maternal origin have been detected in the urine of breast fed infants.[5]

Actions Of Lactoferrin

Lactoferrin is found to play versatile roles in iron absorption, immunity, inflammation, tumour growth, myelin genesis and enzymatic activities as discussed below.

Lactoferin in Iron Homeostasis

Lactoferrin, due to its structural resemblance to transferrin has been implicated in influencing iron homeostasis. This idea came into existence when infants fed exclusively on breast milk (iron content 0.2 to 0.7 mg/l) failed to demonstrate iron deficiency whereas infants fed on formula feeds needed to be supplemented with iron in order to prevent iron deficiency. Although the iron content of breast milk is low, its bioavailabilty is high. This, coupled with the abundant concentration of lactoferrin in breast milk may be responsible for preventing iron deficiency in breast fed infants. Urinary iron concentrations in neonates was found to be corresponding to lactoferrin concentrations in mothers' milk further proving the uptake of lactoferrin by neonates.[64]

The notion that lactoferrin was involved in the absorption of iron was further strengthened by the evidence of receptors specific for lactoferrin on human intestine as established by Cox et al. in 1979.[67] It has been shown that lactoferrin receptors transport iron across the intestinal brush border and facilitate iron accumulation in brush border membrane vesicles.[64] Caco-2 cell lines transfected with lactoferrin receptor have demonstrated an increase by 1.7 to 3.4 times of lactoferrin absorption

compared to mock transfected cells highlighting the unique receptor mediated uptake of iron. [65]

An animal experiment conducted by Kawakami H. et al. in Japan, comparing the efficacy of lactoferrin with ferrous sulphate in improving haematological parameters in iron deficient rats, demonstrated an improvement of parameters in the lactoferrin group.[69] An American study conducted by P.P.Ward et al. involving lactoferrin gene knock out mice failed to demonstrate a decrease in iron absorption. [68] Thus the role of lactoferrin in iron absorption has been equally refuted and proved by independent scientists which has led to considerable debates.

Antimicrobial Effects of Lactoferrin

Lactoferrin exhibits a wide spectrum of antimicrobial actions as demonstrated by numerous studies. The antimicrobial effects of lactoferrin have been attributed to its iron binding property, where the iron required for the microbial survival has been sequestered by lactoferrin. Another mechanism proposed for its antimicrobial actions is the prevention of biofilm formation following iron sequestration. Receptors for N-terminal region of lactoferrin on some gram negative bacteria result in their cell membrane disruption. In gram positive organisms, the positively charged lactoferrin interacts with the negatively charged lipid layer altering the membrane permeability resulting in the bactericidal effect of lactoferrin.[5]

The bactericidal activity of lactoferrin is also evident in the secondary granules of neutrophils where it acts as a source of iron generating free radicals that kill the microbes. Lactoferricin, a peptide degradation product of lactoferrin is considered to possess greater bactericidal activity than lactoferrin.[70]

Anti-viral properties of lactoferrin is evident by its binding to glycosaminoglycans of cell membranes which prevents the entry of viruses such as herpes simplex virus and cytomegalovirus and truncates the infection at an earlier stage.[71] Lactoferrin has also demonstrated anti-parasitic actions against *trichomonas spp, toxoplasma gondii* and *pneumocyctis carinii.*[72]

Role of Lactoferrin in Immunity and Host defence

Lactoferrin is known to increase proliferation, differentiation and activation of the cells of the immune system. It has been shown that a deficiency of lactoferrin is associated with recurrent infections. This may be due to the lack of its actions in neutrophils. Lactoferrin concentrations have been noted to rise by 1000 fold in sepsis. Lactoferrin is also believed to exhibit anti-inflammatory properties. Lactoferrin is known to decrease pro-inflammatory cytokines such as tumour necrosis factor (TNFα), IL-1β and IL-6. [73] Thus the immunomodulatory properties of lactoferrin may prevent inflammatory damage to tissues.

Antineoplastic Actions of Lactoferrin

Lactoferrin is believed to possess antineoplastic actions. This has been demonstrated in animal studies with chemically induced tumours where it has been

reported to inhibit development of metastasis in mice. Lactoferrin mediated increased cytotoxicity of NK cells has been implicated in its antineoplastic effects in epithelial and breast cancers. Low concentrations of lactoferrin have been shown to stimulate the lysis of tumour cells. The anti-tumour property of lactoferrin may also be attributed to its induction of apoptosis, though the exact mechanism is yet to be elucidated.[74]

Lactoferrin as a Growth Factor

Lactoferrin has been implicated in myelopoisis, osteoblast proliferation and lymphocyte proliferation. It has been shown to increase proliferation of rat intestinal crypt cells. Lactoferrin, as a source of iron, facilitates DNA proliferation. It is believed to increase levels of BFU-E and GM-CSF and hence favour myelopoisis and erythropoiesis. Its role in myelopoisis is peppered with discrepancies though reports have failed to demonstrate any inhibitory effect of lactoferrin on myelopoisis.[75]

The action of lactoferrin on osteoblasts and chondrocytes could potentially result in its utility in treating diseases such as osteoporosis. Lactoferrin has demonstrated inhibition of osteoclastogenesis though no effect on bone resorption by mature osteoclasts has been noted. This effect may be due to its inhibition of osteolysis promoting cytokines such as TNFα or IL-1β. [5]

Enzymatic Action of Lactoferrin

Lactoferrin possess structural moieties similar to ribonuclease A and has demonstrated ribonuclease activity. It is believed to be responsible for the DNase

and RNase activity in human milk. In addition, in vitro studies have demonstrated phosphatase and maltoligosaccharide hydrolysis activity. [5]

Preparation of Lactoferrin Available

Lactoferrin is available as lactoferrin fortified bovine colostrum (Laktrum) in India.[Figure 6.] Each gram of Laktrum contains 10mg of lactoferrin in addition to 50 mg of bovine colostrum. Bovine colostrum by itself contains 0.8 mg of bovine lactoferrin per gram of colostrum. 1 mg of bovine lactoferrin can bind approximately 1.4 mg of iron.[62] Bovine colostrum of laktrum is harvested from Biopole Belgian cows within 24 hours and is processed by freeze drying thereby ensuring greater stability of the product. Bovine colostrum also contains 15 mg/g of immunoglobulins and growth factors such as Epithelial Growth Factor, Fibroblast Growth Factor, Platelet Derived Growth Factor, Transforming Growth Factor and Insulin Growth Factor 1 and 2.[76] In addition the product also contains mineral and vitamins in varied concentrations. The excipient added is colloidal silicon dioxide along with sucrose. This product has been in the market for its immunomodulatory and proliferative properties.

Figure 6: Laktrum

Proposed Mechanism of Action of Lactoferrin in Anaemia

The mechanism of how lactoferrin improves anaemia has not been clearly defined. The possible mechanisms are as follows

1. Lactoferrin may act as a source of iron as each holo-lactoferrin molecule contains two ferric ions which can be absorbed via the ITLN-1 receptors present on enterocytes.[67]
2. Lactoferrin may help sequester dietary iron by binding to iron in food and facilitating its uptake by enterocytes via the ITLN-1 receptor.[67] This would be particularly useful in individuals with reduced dietary iron absorption due to defective DMT-1/HCP-1 ferroportin pathway.
3. Lactoferrin has been demonstrated to decrease IL-6 levels. Decreased IL-6 levels result in decreased synthesis of hepcidin, which facilitates increased expression of ferroportin on the basolateral membrane of the enterocytes thereby increasing iron absorption.[77]
4. Lactoferrin is postulated to regulate levels of GM-CSF and BFU-E which in turn could influence myelogenisis and erythropoiesis. However, this action of lactoferrin is debatable due to discrepancies reported by numerous studies.[75]

In addition, there could be other hitherto unknown mechanisms of how lactoferrin improves haematological profiles in iron deficiency anaemia. Due to its iron binding properties, lactoferrin can be explored as an alternative form of therapy for iron deficiency. Hence a study was designed to assess the efficacy of lactoferrin fortified bovine colostrum in treating iron deficiency anaemia in Indian women.

AIM AND OBJECTIVE

Aim

To compare the efficacy of oral lactoferrin fortified bovine colostrum (as a single agent and in combination with ferrous sulphate) with oral ferrous sulphate in treating iron deficiency anaemia.

Objective

To compare the efficacy of oral lactoferrin fortified bovine colostrum (as a single agent and in combination with ferrous sulphate) with oral ferrous sulphate in treating iron deficiency anaemia by measuring haemoglobin levels and iron parameters.

MATERIALS & METHODS

This was a prospective, randomized, active controlled, 3 armed parallel open-labelled comparative study conducted at Govt. Kilpauk Medical College and Hospital between June 2013 and July 2014.

The study procedure required screening for patients with iron deficiency anaemia who satisfy the inclusion criteria, their subsequent recruitment, randomization and grouping, followed by baseline investigations. This was followed by a 30 day treatment regimen and analysis of post therapy investigations which assessed the efficacy of the treatment given.

The Institute Ethical Committee clearance was obtained prior to commencement of the study. The conduct of the study was along the guidelines laid down by ICMR on the conduct of biomedical research.

Sample Size

Results from a previous study conducted on Italian pregnant women was used to calculate sample size as there are no equivalent Indian studies.[77] A two sided t-test was employed to detect superiority of lactoferrin fortified bovine colostrum over ferrous sulphate. A minimum sample size of 12 per group was required to have a 90% chance (alpha error of 0.05) of detecting an increase in haemoglobin level to 11.5 g/dl (S.D of 0.6 g/dl) in the ferrous sulphate group and

12.7g/dl (S.D of 0.9 g/dl) in the experimental group. On adjustment for non-compliance in the ferrous sulphate arm to 40%, a sample size of 68 was arrived at.

Screening

Four medical camps were organized by the principal investigator amongst nursing students belonging to the School of Nursing, Govt. Kilpauk Medical College and Hospital, Chennai. Nursing students were chosen as a cohort in order to minimize confounding factors with the added advantage of age-matched counterparts in each group.

During the medical camp, conjunctival pallor was assessed clinically. Those found to be pale were advised to get their haemoglobin and peripheral smear assessed.

Recruitment

Students fulfilling the inclusion criteria were briefed on the study. Written informed consent was obtained from those willing to participate in the study. A detailed history and clinical examination was performed on the participants. Venous blood was drawn for baseline assessment of iron parameters and other routine tests.

Grouping

The study design envisaged a randomization of the study population into 3 arms. The control arm received oral Ferrous Sulphate 333mg (100mg elemental iron) once daily. The study arm received oral Lactoferrin Fortified Bovine

Colostrum 2g once daily. The third arm received both Ferrous Sulphate and Lactoferrin Fortified Bovine Colostrum.

Table 4: Grouping of Study Participants

Group I	Group II	Group III
n=25	n=25	n=18
Oral Ferrous sulphate	Oral Lactoferrin Fortified Bovine Colostrum	Oral Ferrous sulphate and Lactoferrin Fortified Bovine Colostrum

Drugs Used in the Study and Their Sources

1. Tab. Ferrous Sulphate 333 mg (100 mg of elemental iron) once daily to be taken on empty stomach. This drug was sourced from Govt. Kilpauk Medical College and Hospital, Chennai.

2. Oral Laktrum 2g once daily to be taken on an empty stomach. This nutraceutical was supplied by 'Tablets India', Chennai.

Both supplements were administered orally for a period of 30 days. Lactoferrin Fortified Bovine Colostrum was supplied as a powder and had to be reconstituted in 200 ml of potable water before consumption.

Randomization

A simple randomization method was adopted using Microsoft Excel. Random numbers between 0 to 1 (up to 4 decimal points) were generated. The participant enrollment number was arranged numerically (1 to 68).A filter was applied to arrange the random numbers in the ascending order. The first 25 numbers were assigned as controls (Group I – Ferrous Sulphate Arm). The next 25 numbers were assigned to the Test group (Group II – Lactoferrin Fortified Bovine Colostrum Arm) and the last 18 numbers were assigned to the group receiving both the test and the control supplements (Group III – Combination Therapy Arm).

The participant enrollment number was assigned in running number as and when a student was found suitable for recruitment into the study.

Inclusion Criteria

A Student was considered suitable for the study if she satisfied the following inclusion criteria:

a. Females above the age of 18 years.

b. Presence of hypochromic microcytic anaemia.

c. Haemoglobin level between 8 and 11 mg/dl.

Exclusion Criteria

Students with the following criteria were excluded from the study:

a. Female patients under 18 years of age or Male patients.
b. Non-microcytic hypochromic anaemia.
c. Haemoglobin levels more than 11 mg/dl or less than 8 mg/dl.
d. Known cases of anaemia due to secondary causes such as infections, neoplasms, genetic diseases, hypochlorhydria or renal disease
e. Presence of inflammation or infection detectable by a raised ESR (associated with increased ferritin levels leading to false negatives).
f. Patients on any medication that could interfere with iron absorption such as antacids/ PPIs.
g. Patients on iron supplements or a history of recent supplementation (past 3 months).
h. Patients having menorrhagia or a known source of bleeding (varices, ulcers etc.).
i. Patients requiring blood transfusion or with recent history of blood transfusion.
j. Seriously ill patients

Students who fit the inclusion criteria and were willing to participate in the study were recruited into the study.

Assessment of Participants

A clinical assessment was performed (as per annexure II) after obtaining a detailed history. The findings were duly noted in the case report forms (CRF).

8ml of venous blood was collected from the subject under strict aseptic precautions for routine investigations such as:

Complete Blood count (TC, DC, ESR, Haemoglobin)

Peripheral Smear

Sr. Urea

Sr. Creatinine

Sr. Bilirubin

SGOT/PT

Assessment of Iron Status of the Participants

Sample for iron studies was aliquoted and made to rest for a minimum of one hour after which it was centrifuged at 3000 r.p.m for four minutes. The serum was separated and aliquoted into 3 separate eppendorfs after appropriate labelling.

The eppendorf with the sera were stored in the deep freezer at -20 degrees centigrade for running the following tests at a later date:

1. Sr. Iron
2. Sr. Ferritin
3. Total Iron binding capacity
4. Sr. Transferrin Saturation (to be calculated from above)
5. Unsaturated Iron Binding capacity (to be calculated from above)

I. Quantitative Estimation of Serum Iron

Serum iron concentration was measured by end-point colorimetric assay using FERROZINE (Spinereact Ref: 1001247, 1001248).

Principle of the Test

Iron dissociated from transferrin by provision of an acidic medium and reduced to ferrous form by addition of ascorbic acid. This reduced iron forms a coloured complex in combination with ferrozine, the intensity of which is measured using colorimetric analysis and is indicative of serum iron concentration.

Colorimetric analysis has been a reliable, reproducible and sensitive method of assaying serum iron concentration over 20 years. The specificity of the test was enhanced by blanking where every test samples is paired and reagent added to only one of the pairs.[Figure 7.] This method of blanking facilitates the measurement of

the baseline absorbance of the samples which can be subtracted from its paired reading to provide accurate serum iron concentration values.

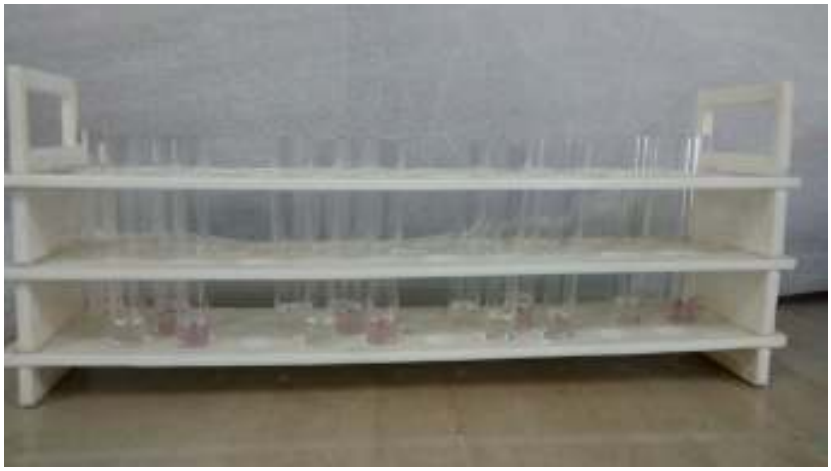


Figure 7: Blanking Procedure for Serum Iron Concentration Analysis

Materials Required

1. Ferrozine kit (1 No.) containing
    i. 100 mmol/L of acetate at pH 4.9 that acts as a buffer
    ii. Ascorbic acid (99.7%)
    iii. 40 mmol/L FerroZine color
    iv. 100 ug/dl of aqueous iron as standard
2. Colorimeter measuring at 562 nm
3. 100 mcl, 200mcl, 1000mcl micropipettes with disposable micropipette tips , test tubes

Procedure

1. Working reagent was prepared by adding one tube of ascorbic acid to one bottle of buffer and mixed gently to dissolve the contents.
2. The instrument was calibrated to read at 562 nm with a 1 cm cuvette light path at room temperature. Instrument was adjusted to zero with distilled water.
3. Test tubes were paired and labelled. A working reagent (WR) blank and standard were tested with every batch that is processed.
4. Reagents were added as presented below [Table 5.]:

Table 5: Reagents Used In Serum Iron Assay

Components	WR Blank	Standard	Sample Blank	Sample
WR (ml)	1	1	1	1
Ferrozine (drops)	1	1	-	1
Distilled water (microliter)	200			
Standard (microliter)		200		
Sample (microliter)			200	200

5. Contents of each test tube were allowed to mix and incubate for 10 min at room temperature
6. The absorbance [A] of standard and sample against WR blank was measured using a colorimeter after calibration.
7. The concentration of sample iron was calculated as follows:
8. Serum Iron Concentration (mcg/dl) = $\frac{[A]\ \text{Sample} - [A]\ \text{Sample Blank}}{[A]\ \text{Standard}} \times 100$

II. Quantitative Estimation of Serum Ferritin

Serum ferritin was assayed by an enzyme-immunoassay (EIA) validated for quantitative in-vitro determination of ferritin in human serum. 2 kits of PATHOZYME FERRITIN (Ref OD407) were procured for the same.

Principle of the Test

The test is based on the general principle of an Enzyme linked immunoassay. Specific anti ferritin antibodies coated onto the micro titration wells, bind to ferritin in the test sera. A conjugate of a monoclonal anti-ferritin labelled with horseradish peroxidase enzyme is then made to bind to the ferritin. On addition of the substrate, a development of change in colour indicates the presence of ferritin. The intensity of the colour quantified by colorimetric analysis, translates to the concentration of ferritin in the test sera.

Materials Required

1. 100 mcl, 200mcl, 1000mcl micropipettes with disposable micropipette tips
2. Absorbent paper
3. Micro plate reader with a 450 nm filter
4. Micro plate washer

5. 2 kits of PATHOZYME [R] FERRITIN (Ref OD407) with the following contents:

   i. 12x8x1 breakable wells coated with a specific antibody

   ii. 0.5 ml of 6 reference standards of 0ng/ml, 15ng/ml, 80ng/ml, 250ng/ml, 500ng/ml and1000ng/ml of ferritin diluted in human serum

   iii. 11 ml of anti-ferritin Horse radish peroxidase conjugate

   iv. 11 ml of substrate solution 3,3',5,5' Tetra methyl benzidine in citrate buffer

   v. 11ml of dilute hydrochloric acid as stop solution

Assay Procedure

1. The kit components were all brought to room temperature of 20-25 degree Celsius prior to the start of the assay and were mixed thoroughly by gentle inversion.
2. The test sera were arranged and labelled in running numbers.
3. 6 wells were designated for standardization per batch.
4. 100 microliters of anti-ferritin was dispensed in each well carefully without touching the walls of the well.
5. 20 micro litres of the standard and test sera were dispensed into the wells using a micro pipette.

6. 100 micro litres of horseradish peroxidase enzyme conjugate was instilled into all the wells.

7. The three components were thoroughly mixed in their respective wells by gentle rotatory movements of the micro titre plate.

8. The plate was then allowed to incubate for 45 min at room temperature.

9. The contents of the micro titre plate were then discarded and were placed in the micro plate washer which was programmed to instil 300 micro litres per cycle and run for 5 cycles.

10. The plate was removed from the washer and any residual water removed by sharply striking the plate onto absorbent blotting paper.

11. 100 micro litres of the substrate was added to each well, mixed and allowed to incubate for 20 minutes. Care was taken not to expose the wells to direct sunlight.

12. 100 micro litres of stop solution was added to each well to stop further reactions which was indicated by the change in colour from blue to yellow.

13. The optical density of each well was read with using a micro plate reader with a 450 nm filter calibrated to a quadratic regression curve fit.

III. Quantitative Estimation Of Total Iron Binding Capacity (TIBC)

TIBC was measured by end point analysis by saturation- precipitation method. 2 kits of Spinereact TIBC (Ref: 1001241) were procured for the same.

Principle of the Test

Serum transferrin is saturated with excess ferric ion and precipitation of unbound iron induced by the addition of magnesium carbonate. After centrifugation, the iron in the supernatant is measured using endpoint calorimetry method as described for serum iron assay.

Materials

1. TIBC kit containing
    a. 500 mcg/dl of iron solution
    b. Magnesium carbonate
2. Ferrozine kit with contents as described for serum iron assay
3. Colorimeter measuring at 562 nm
4. 100 mcl, 200mcl, 1000mcl micropipettes with disposable micropipette tips , test tubes

Procedure

1. Samples arranged in order were assigned running numbers and labelled.

2. To 0.5 ml of sample, 1 ml of iron solution was added, mixed well and allowed to incubate at room temperature for 10 min.

3. 3 spoonsful (~ 70 mg) of precipitating reagent were added to each tube, mixed well and allowed to incubate for 10 min at room temperature.

4. The contents of the test tubes were then centrifuged for 15 minutes at 3000 revolutions per minute

5. The supernatant was collected carefully and iron content measured.

6. TIBC was calculated as follows:

   TIBC= Iron concentration in supernatant X 3 (dilution factor)

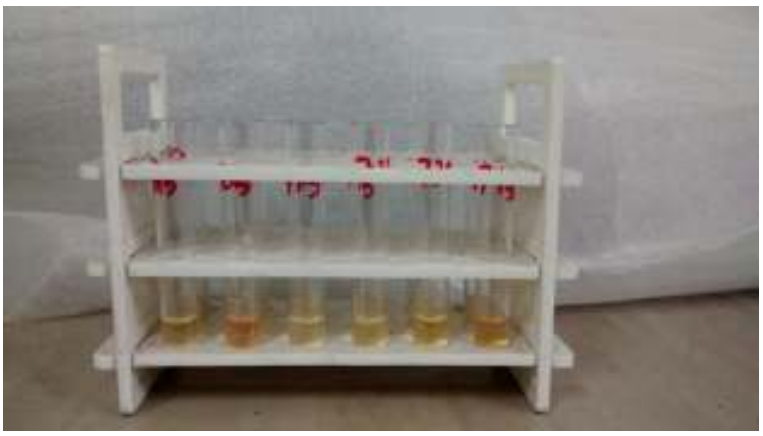


Figure 8: Precipitation of Unbound Iron in TIBC Assay

IV. Calculation of Unsaturated Iron Binding Capacity

Unsaturated Iron Binding Capacity was calculated as follows:

UIBC (mcg/dl) =TIBC- Sr.Iron

V. Calculation of Transferrin Saturation (TSAT)

Transferrin Saturation was calculated parameter as follows:

$$\text{TSAT \%} = \frac{\text{Serum iron concentration}}{\text{TIBC}} \times 100$$

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 17 software. Continuous variables were to be described as means along with their standard deviations while discrete variables were expressed as frequencies and percentages. Distributions for continuous variables were assessed for their linearity. Within each group, the mean change in haemoglobin and iron parameters from baseline to post-therapy was assessed using student's paired t-test. A one way ANOVA was employed to assess the significance of change in parameters between the three groups. A non-parametric Wilcoxon signed rank test was employed for the analysis of serum ferritin. A descriptive analysis was undertaken for the analysis of Adverse Drug Reactions that occurred during the study period.

RESULTS

A total of 242 students were screened for the clinical presence of conjunctival pallor. A complete blood count and peripheral smear was undertaken on 84 students who were found to exhibit clinical pallor.[Figure 9] 16 of them did not fit the inclusion criteria and were excluded from the study. The 68 students recruited into the study were randomly allotted into three groups as depicted in Table 4.

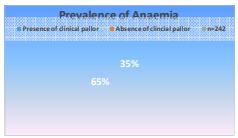


Figure 9: Pie Chart of Prevalence of Anaemia

Demographics

As per the inclusion criteria, all the participants of the study belonged to the female gender. The mean age of the study participants was found to be 19.15 ± 0.685 years ranging from 18 to 21 years. There was no significant variation in age within group or between the three groups.

Routine Investigations

The baseline and post-treatment values of TC, DC, ESR, SGOT, SGPT, Sr. Urea, Sr. Creatinine and Sr.Bilirubin were found to be normal in all the subjects.

Table 6: Group I (Ferrous Sulphate Arm) Descriptive Statistics

Parameters	Sample	Minimum	Maximum	Mean	S.D
Haemoglobin (g/dl)	Baseline	8	11	9.5191	0.76271
Haemoglobin (g/dl)	Post-therapy	7.8	12.5	10.1391	0.91135
Sr. Ferritin (mcg/dl)	Baseline	0.399	136.1	19.87175	25.50824
Sr.Ferritin (mcg/dl)	Post-therapy	2.653	191.5	20.60839	27.60475
Sr.Iron (mcg/dl)	Baseline	8.82	106.7	48.4379	22.42331
Sr.Iron (mcg/dl)	Post-therapy	12.59	164.74	60.2364	23.24625
Sr.TIBC (mcg/dl)	Baseline	321.78	694.35	511.1379	76.84376
Sr.TIBC (mcg/dl)	Post-therapy	260.07	678.63	438.3676	72.13451
Sr. UIBC (mcg/dl)	Baseline	236.12	649.46	462.7	81.11258
Sr.UIBC (mcg/dl)	Post-therapy	157.46	613.75	378.1312	74.48859
Sr.TSAT (%)	Baseline	1.75	19.13	9.9287	4.51106
Sr. TSAT (%)	Post-therapy	2.77	25.15	10.7201	5.33559

Table 7: Group I (Ferrous Sulphate Arm) Paired t-Test

Change in Parameter	Mean	95% Confidence Interval		p-Value
		Lower	Upper	
Δ Haemoglobin	0.028	-0.12942	0.18542	0.717
Δ Sr. Ferritin	-0.71956	-5.3926131	3.9534931	0.753
Δ Sr. Iron	1.544	-8.4068	11.4948	0.752
Δ TIBC	-16.8732	-48.86705	15.12065	0.287
Δ UIBC	-18.4172	-54.79935	17.96495	0.307
Δ TSAT	0.79140	-1.47008	3.05289	0.477

* $p<0.05$ is considered significant

Table 8: Group II (Lactoferrin Fortified Bovine Colostrum Arm) Descriptive Statistics

Parameters	Sample	Minimum	Maximum	Mean	S.D
Haemoglobin (g/dl)	Baseline	8	11	9.4784	0.83044
Haemoglobin (g/dl)	Post-therapy	8	12.5	10.4072	1.08726
Sr. Ferritin (mcg/dl)	Baseline	0.666	136.1	20.11496	28.36195
Sr.Ferritin (mcg/dl)	Post-therapy	2.84	153.17	20.81648	30.84125
Sr.Iron (mcg/dl)	Baseline	17.34	80.66	48.272	18.82853
Sr.Iron (mcg/dl)	Post-therapy	16.94	164.74	66.7672	39.40832
Sr.TIBC (mcg/dl)	Baseline	372.99	681.87	510.504	81.48118
Sr.TIBC (mcg/dl)	Post-therapy	302.49	621.39	392.328	78.60037
Sr. UIBC (mcg/dl)	Baseline	315.09	625.68	462.232	83.00612
Sr.UIBC (mcg/dl)	Post-therapy	157.46	548.79	325.5608	93.00596
Sr.TSAT (%)	Baseline	3.08	16.22	9.6780	3.95709
Sr. TSAT (%)	Post-therapy	4.62	50.56	17.8374	11.50182

Table 9: Group II (Lactoferrin Fortified Bovine Colostrum Arm) Paired t-Test

Parameters	Mean	95% Confidence Interval		p-Value
		Lower	Upper	
Δ Haemoglobin*	0.9288	0.5634	1.2942	<0.01
Δ Sr. Ferritin	0.701521	-4.6488118	6.0518534	0.789
Δ Sr. Iron*	18.4952	4.40917	32.58123	0.012
Δ TIBC*	-118.176	-157.64586	-78.70614	<0.01
Δ UIBC*	-136.671	-185.53979	-87.80261	<0.01
Δ TSAT*	8.15946	3.67436	12.64456	0.01

* $p<0.05$ is considered significant

Table 10: Group III (Combination Therapy Arm) Descriptive Statistics

Parameters	Sample	Minimum	Maximum	Mean	S.D
Haemoglobin (g/dl)	Baseline	8	11	9.0562	0.98791
Haemoglobin (g/dl)	Post-therapy	8.1	11.5	10.1187	0.8916
Sr. Ferritin (mcg/dl)	Baseline	1.803	91.65	25.42547	27.98601
Sr.Ferritin (mcg/dl)	Post-therapy	3.852	191.5	28.49009	46.87105
Sr.Iron (mcg/dl)	Baseline	10.79	92.5	44.4869	23.69757
Sr.Iron (mcg/dl)	Post-therapy	14.86	112.6	61.8444	26.74477
Sr.TIBC (mcg/dl)	Baseline	321.78	686.7	492.8187	98.28453
Sr.TIBC (mcg/dl)	Post-therapy	260.07	678.63	403.6556	101.6775
Sr. UIBC (mcg/dl)	Baseline	236.12	649.46	448.3319	108.9829
Sr.UIBC (mcg/dl)	Post-therapy	175.25	606.08	341.8113	110.5557
Sr.TSAT (%)	Baseline	2.07	16.63	9.2842	5.20309
Sr. TSAT (%)	Post-therapy	3.25	33.27	16.9779	8.55587

* $p<0.05$ is considered significant

Table 11: Group III (Combination Therapy Arm) Paired t-Test

Parameters	Mean	95% Confidence Interval		p-Value
		Lower	Upper	
Δ Haemoglobin *	1.0625	0.52544	1.59956	0.001
Δ Sr. Ferritin	3.064625	-11.9268783	18.0561283	0.669
Δ Sr. Iron *	17.3575	4.50649	30.20851	0.011
Δ TIBC*	-89.1631	-153.66176	-24.66449	0.01
Δ UIBC*	-106.521	-173.23954	-39.80171	0.004
Δ TSAT*	7.69367	3.66723	11.72011	0.01

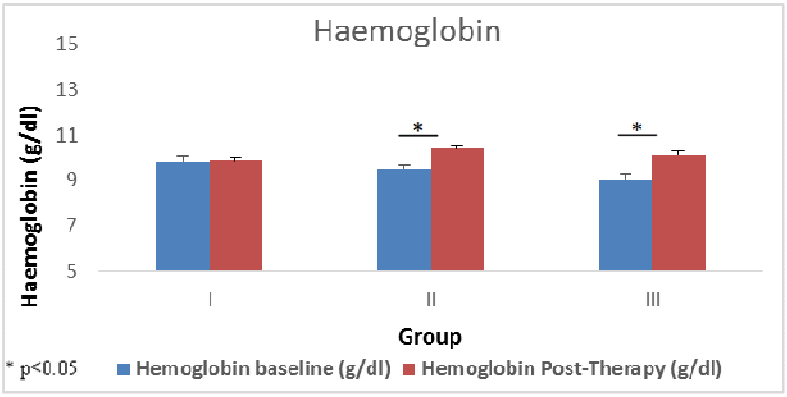


Figure 10: Bar Diagram of Serum Haemoglobin

Student's paired t-test for Haemoglobin

Participants in Group I (Ferrous Sulphate Arm) showed a mean increase in haemoglobin of 0.028 g/dl which was not found to be statistically significant ($p = 0.717$).

Participants in Group II (Lactoferrin Fortified Bovine Colostrum Arm) showed a significant increase in their haemoglobin levels post-therapy with a mean increase in haemoglobin of 0.9288 g/dl ($p<0.001$)

Participants in Group III (Combination Therapy Arm) also showed a significant increase in their haemoglobin levels post-therapy with a mean increase in haemoglobin of 1.0625 g/dl ($p=0.001$).

ANOVA and Multiple Comparison Test Analysis of Haemoglobin

There was no significant difference in baseline values between the three groups. However, a significant difference in the rise in haemoglobin values in Group II and III ($p <0.001$ and 0.001 respectively) was noted in comparison to Group I ($p = 0.717$). There was no significant difference in the rise in haemoglobin between Group II and Group III ($p = 0.877$).

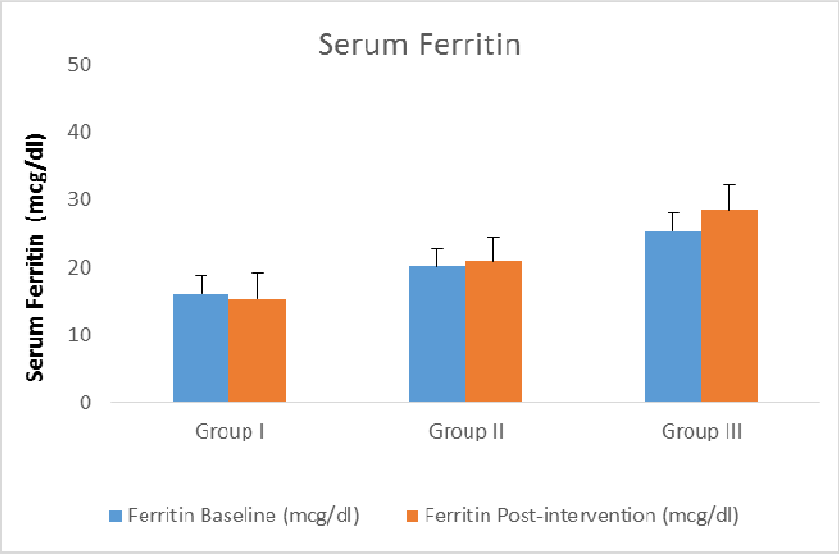


Figure 11: Bar Diagram of Serum Ferritin

Wilcoxon Signed-Rank Test for Serum Ferritin

It was found that ferritin followed a non-normal distribution. Hence a non-parametric Wilcoxon signed-rank test was employed to analyse serum ferritin levels.

A Wilcoxon signed-rank test demonstrated a decrease in ferritin stores in participants of Group I (Ferrous Sulphate Arm) that was not found to be statistically significant ($p = 0.420$, $z = 0.807$).

Participants of Group II (Lactoferrin Fortified Bovine Colostrum Arm) demonstrated an increase in ferritin levels post-therapy which was again found to be statistically insignificant ($p = 0.819$, $z = 0.229$).

Participants of Group III (Combination Arm) demonstrated an increase in ferritin levels post-therapy which was also found to be statistically insignificant ($p = 0.717$, $z = 0.362$).

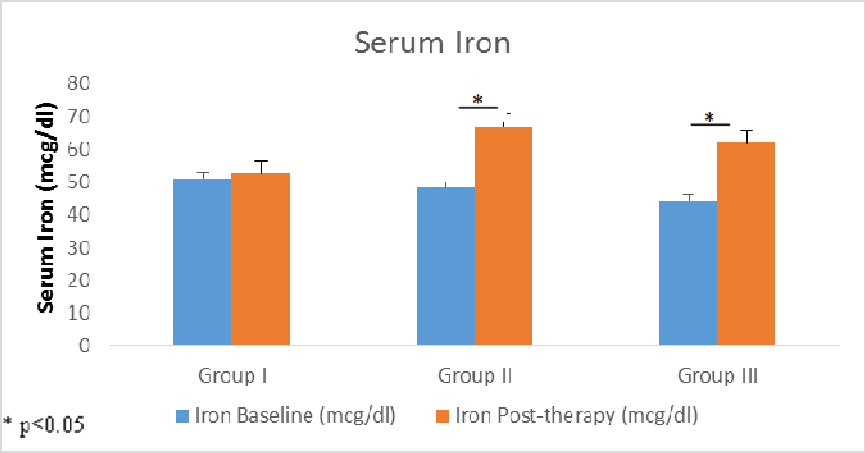


Figure 12: Bar Diagram of Serum Iron

Student's Paired t-test for Serum Iron Concentration

Participants in Group I (Ferrous Sulphate Arm) showed a mean increase in serum iron concentration of 1.544 mcg/dl which was not found to be statistically significant (p=0.752).

Participants in Group II (Lactoferrin Fortified Bovine Colostrum Arm) showed a significant increase in their serum iron concentration values post-therapy with a mean increase in serum iron concentration of 18.4952 mcg/dl (p= 0.012).

Participants in Group III (Combination Therapy Arm) also showed a significant increase in their serum iron concentration values post-therapy with a mean increase in serum iron concentration of 17.3575 mcg/dl (p=0.011).

ANOVA and Multiple Comparison Test Analysis of Serum Iron Concentration

There was no significant difference in baseline values of serum iron concentration between the three groups. There was no significant difference in the rise in serum iron concentration levels between the three groups (p = 0.095,0.198, 0.991).

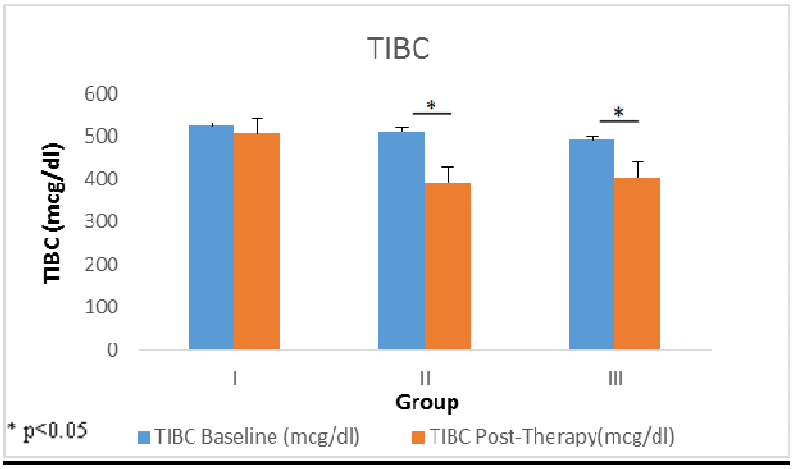


Figure 13: Bar Diagram of TIBC

Student's paired t-test for Total Iron Binding Capacity (TIBC)

Participants in Group I (Ferrous Sulphate Arm) showed a mean decrease in TIBC of 16.8732 mcg/dl which was not found to be statistically significant (p= 0.287).

Participants in Group II (Lactoferrin Fortified Bovine Colostrum Arm) showed a significant decrease in TIBC values post-therapy with a mean decrease in TIBC of 118.176 mcg/dl ($p < 0.001$).

Participants in Group III (Combination Therapy Arm) also showed a significant decrease in their TIBC values post-therapy with a mean decrease in TIBC of 89.16313 mcg/dl ($p = 0.01$).

ANOVA and Multiple Comparison Test Analysis of Total Iron Binding Capacity (TIBC)

There was no significant difference in baseline values of TIBC between the three groups.

Post-therapy TIBC values of Group II and III were significantly different from that of Group I ($p<0.01$, $p = 0.01$ respectively). There was no significant difference in the post-therapy TIBC values between Group II and III ($p = 0.904$).

When a difference in difference analysis was carried out, the fall in TIBC values of Group II were significantly more than that observed in Group I ($p = 0.001$).

There was no significant difference in change in TIBC values observed between Group I and III ($p = 0.057$) or between Group II and III ($p= 0.616$)

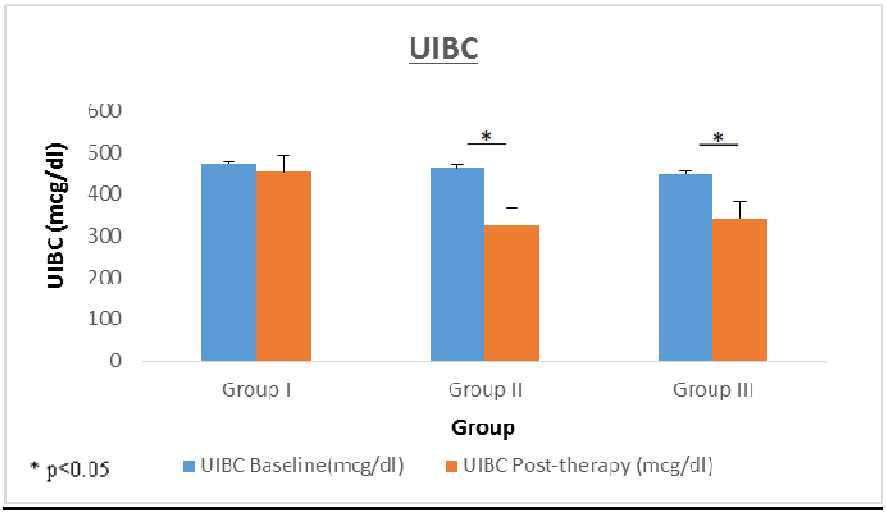


Figure 14: Bar Diagram of UIBC

Student's Paired t-test for Unsaturated Iron Binding Capacity (UIBC)

Participants in Group I (Ferrous Sulphate Arm) showed a mean decrease in UIBC of 18.4172 mcg/dl which was not found to be statistically significant ($p = 0.307$).

Participants in Group II (Lactoferrin Fortified Bovine Colostrum Arm) showed a significant decrease in UIBC values post-therapy with a mean decrease in UIBC of 136.6712 mcg/dl ($p < 0.001$)

Participants in Group III (Combination Therapy Arm) also showed a significant decrease in their UIBC values post-therapy with a mean decrease in UIBC of 106.521mcg/dl ($p = 0.004$)

ANOVA and Multiple Comparison Test Analysis of Unsaturated Iron binding Capacity (UIBC)

There was no significant difference in baseline values of UIBC between the three groups.

Post-therapy the UIBC values of Group II and III were significantly different from that of Group I ($p < 0.01$, $p = 0.01$ respectively). There was no significant difference in the post-therapy UIBC values between Group II and III ($p = 0.844$)

When a difference in difference analysis was carried out, the fall in UIBC values of Group II and III were significantly more than that observed in Group I ($p = 0.001, 0.039$ respectively)

There was no significant difference in change in UIBC values observed between Group II and III ($p= 0.668$)

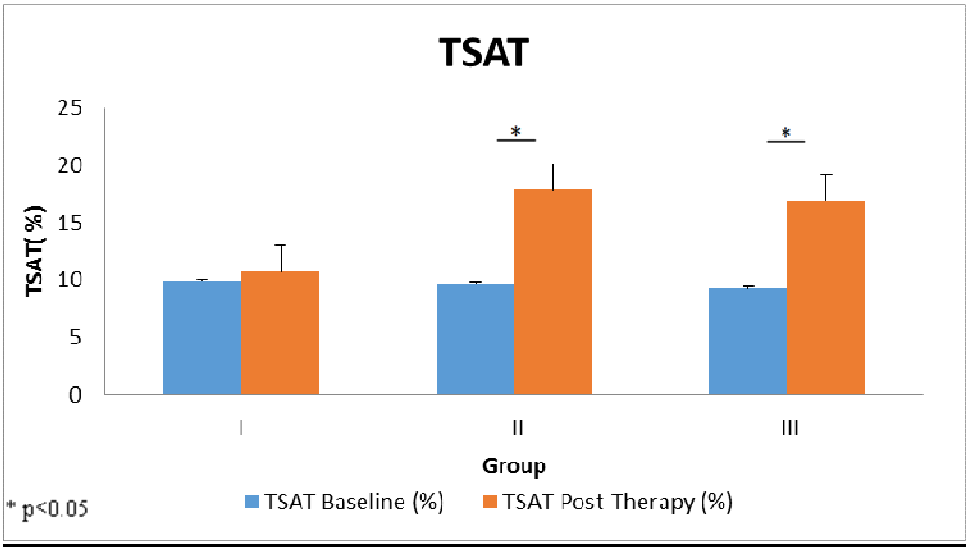


Figure 15: Bar Diagram of TSAT

Student's paired t-test for Transferrin Saturation (TSAT)

Participants in Group I (Ferrous Sulphate Arm) showed a mean increase TSAT of 0.79140% which was not found to be statistically significant (p = 0.477).

Participants in Group II (Lactoferrin Fortified Bovine Colostrum Arm) showed a significant increase in TSAT values post-therapy with a mean increase in TSAT of 8.15946% (p = 0.001).

Participants in Group III (Combination Therapy Arm)also showed a significant increase in their TSAT values post-therapy with a mean increase in TSAT of 7.69367% (p = 0.001).

ANOVA and Multiple Comparison Test Analysis of Transferrin Saturation (TSAT)

There was no significant difference in baseline values of TSAT between the three groups.

Post-therapy TSAT values of Group II was significantly different from that of Group I ($p = 0.017$). There was no significant difference in the post-therapy TSAT values between Group II and III or between Group I and III ($p = 0.951, 0.078$ respectively).

When a difference in difference analysis was carried out, the rise in TSAT values of Group II and III were significantly more than that observed in Group I ($p = 0.008, 0.03$ respectively).

There was no significant difference in change in TSAT values observed between Group II and III ($p = 0.983$).

ADVERSE DRUG REACTIONS

12 participants reported adverse effects to their medication. Most of the adverse effects were not reported spontaneously. They were reported on questioning the participants at the end of the study period. The only exception was a single case of gastritis who was treated with H_2 blocker during the study period. The group-wise split up is presented in the bar diagram below.[Figure 16.]

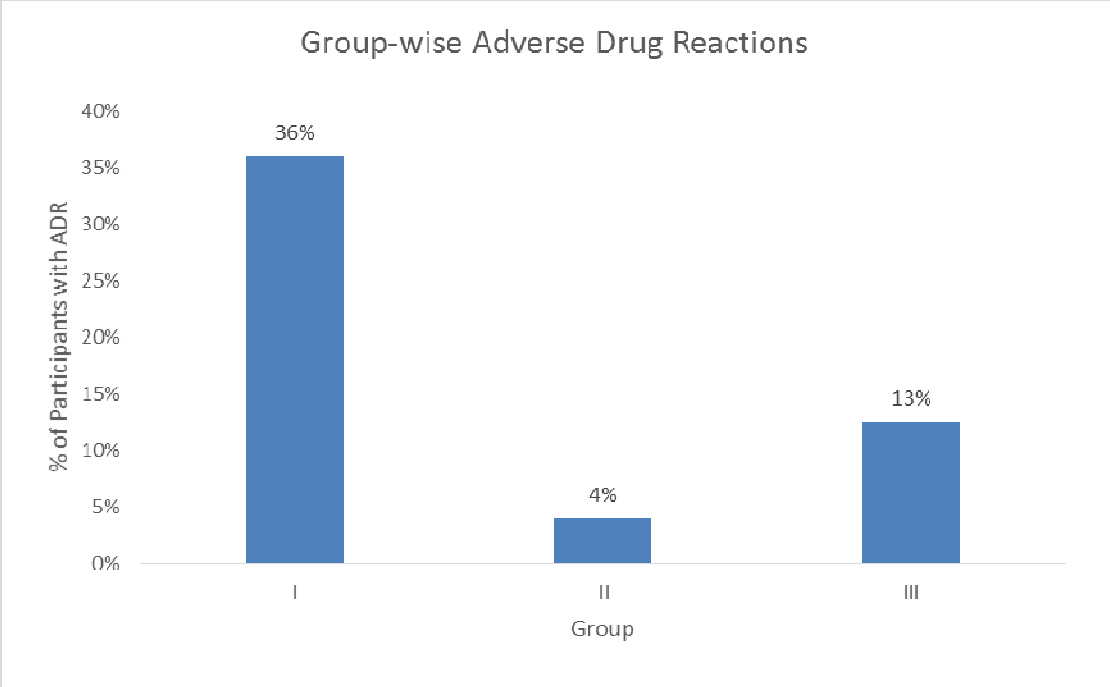


Figure 16: Bar Diagram of Group-wise Adverse Drug Reactions

Common Adverse Drug Reactions (ADR) Reported

Gastritis, constipation, nausea, vomiting and loose stools were the side effects showed in the participants during the study duration.

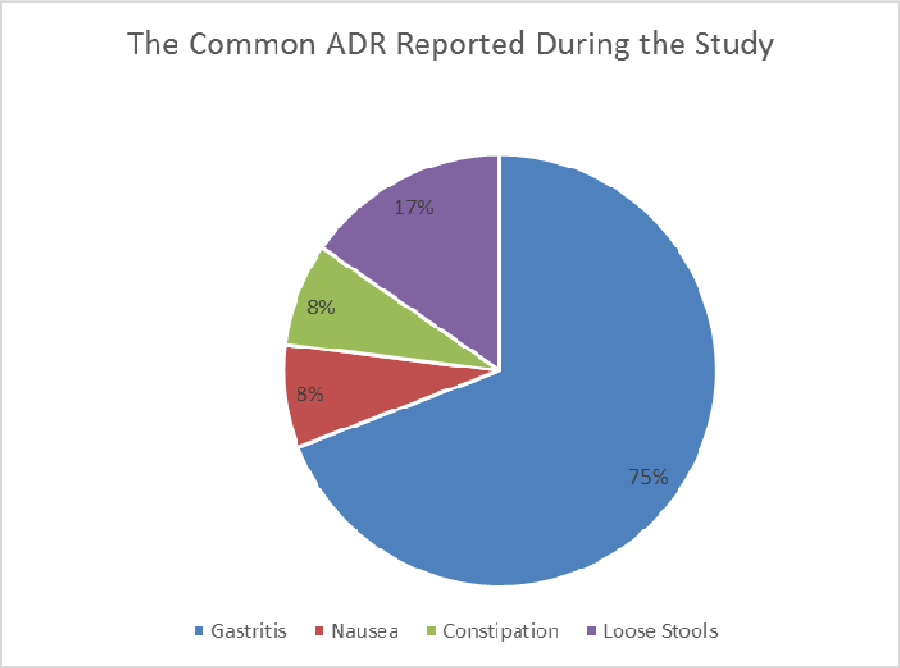


Figure 17: Pie Chart of Common ADR Reported

DESCRIPTION OF ADVERSE DRUG REACTIONS REPORTED

Group I (Ferrous Sulphate Arm)

9 participants reported adverse drug reaction to their medication. 7 participants reported gastritis with 2 of them missing 3-4 doses of their medication. The remaining 5 had mild bearable gastritis. One of them reported mild gastritis and constipation during the first week of treatment. Two other participants reported missing their doses for 5 and 3 days dues to constipation and nausea respectively.

Group II (Lactoferrin Fortified Bovine Colostrum Arm)

There were no incidents of gastritis or constipation although one participant complained of repeated loose stools a few hours after ingesting her medication. There was no increase in frequency of stools. A possibility of lactose intolerance was suspected in this participant. The participant did not discontinue her medication.

Group III (Combination Therapy Arm)

Two participants reported occurrence of mild to moderate gastritis, one of whom was prescribed T.Ranitdine 20 mg BD concomitantly for a period of 2 weeks. There were no reports of change in bowel habits in the participants of this group.

ADHERENCE TO TREATMENT REGIMEN

Of the 68 participants who were recruited in the study, 66 completed the study. The two drop outs belonged to Group III (Combination Therapy Arm). One was diagnosed with infective hepatitis and was not available for follow-up. The other drop-out resorted to intravenous iron instead of taking her oral medications and voluntarily dropped out of the study.

Participants were interviewed on their adherence to their 30 day treatment regimen. The compliance in Group I (Ferrous Sulphate Arm) was found to be 44% which is much lower than that found in the arms recieveing lactoferrin fortified bovine colostrum. The compliance in Group II and III were both found to be 94%.

DISCUSSION

Oral ferrous sulphate is the most commonly prescribed drug for treating iron deficiency anaemia. The poor compliance, GI adverse drug reactions and the variable bioavailability of oral ferrous sulphate emphasize a need for a better oral formulation. Lactoferrin, a glycoprotein structurally resembling transferrin, is believed to play a role in iron absorption. Previous studies conducted in Italian pregnant women and Japanese athletes have shown beneficial effects of bovine lactoferrin in treating iron deficiency anaemia. Hence, a randomized, active controlled, open-labelled, 3 armed parallel study was designed to compare the efficacy of oral lactoferrin fortified bovine colostrum (as single agent and in combination with ferrous sulphate) with oral ferrous Sulphate in treating iron deficiency anaemia.

The prevalence of anaemia amongst nursing students in our study was found to be 35%. This is comparable to previous studies conducted in India, that have stated that at least 40% of asymptomatic nurses in their study had biochemical evidence of iron deficiency anaemia.[23,79]

Our results show significant improvement in haemoglobin and most iron parameters in participants of Group II (Lactoferrin Fortified Bovine Colostrum Arm) and Group III (Combination Therapy Arm) compared to Group I (Ferrous Sulphate Arm).

The significant increase in haemoglobin and serum iron concentration values following therapy in both Group II and III ($p < 0.05$) is in accordance with a previous study conducted among pregnant women in Italy in 2006 by Paesano et al who reported a mean rise in haemoglobin of 1.5g/dl and a mean increase in serum iron concentration of 54.2 mcg/dl.[78] Our study showed a mean rise in haemoglobin of 0.9288g/dl and a mean rise in serum iron concentration of 18.4952 mcg/dl in Group II.[Table 9] Group III showed a mean rise in haemoglobin of 1.0625g/dl and mean rise in serum iron concentration of 17.3575mcg/dl.[Table 11] The mean rise in haemoglobin and serum iron concentration in our study is lower compared to the Italian study which may be explained by the higher dose of lactoferrin employed in the latter study. Though our rise in haemoglobin and serum iron concentration values are lower than their study [78], the rise observed in Group II and III are both clinically and statistically significant. ($p < 0.05$)

The participants in the ferrous sulphate arm failed to register improved haemoglobin levels and iron parameters. This is in accordance with a study conducted in Italian pregnant women in 2010 who were prescribed 156 mg of elemental iron for 30 days. This study documented a parallel fall in serum IL-6 levels which may lead to a hepcidin mediated down regulation of ferroportin resulting in decreased enteral iron absorption. [77]

The rise in haemoglobin (0.028g/dl) in Group I much less than a previous study (1.05 g/dl) conducted among pregnant women in India in the Guntur District by Chandrakala Kambar et al.[80] This disparity may be attributed to better compliance and longer duration of therapy amongst the pregnant women.

On the contrary, the mean rise in serum iron concentration in our study (1.544 mcg/dl) is thrice that reported by their study (0.5 g/dl).[2] This may be due to increased utilization of iron resulting in minimal rise in serum iron concentration in pregnant women of that study.

The marginal rise in haemoglobin (0.028 g/dl, $p = 0.717$) and serum iron concentration (1.544 mcg/dl, $p = 0.752$) in Group I was significantly lower compared to Group II and III ($p < 0.05$). Hence it is evident that lactoferrin fortified bovine colostrum has resulted in a rise in serum iron concentration that has translated into improved haemoglobin levels.

The change in ferritin levels following therapy is not statistically significant in all the three groups. This may be due to the fact that at least a three month course of therapy is required for restoring iron stores in the body.[81] Ferritin levels have been noted to rise as early as 2 weeks in an ICMR study conducted by Alka Kriplani et al., where the route of administration was found to be intravenous which has a higher bioavailability than the oral route.[27] Another study conducted by Natsue Koikawa et al amongst Japanese runners, comparing efficacy of lactoferrin and oral ferrous sulphate reported a decrease in ferritin stores in both the arms after 8 weeks of therapy. [82] Our study was designed along the lines of the Italian study conducted by Pasaeno et al., who recommended a one month course of oral lactoferrin. Hence as our study was designed for a period of one month, and as the route of therapy was oral, the change in ferritin is along the expected lines.

Serum iron concentration has an inversely proportional relationship with TIBC and UIBC and hence the marginal rise in serum iron concentration in the ferrous sulphate arm is only associated with a marginal fall in TIBC and UIBC.[86] The significant fall in TIBC and UIBC observed in the test arm and the combination arm is in agreement with a previous Italian study by Nappi et al. [83] The Italian study documented significant fall in TIBC even in the ferrous sulphate arm which also demonstrated significant rise in serum iron concentration.

Group II and Group III demonstrated a significant rise in transferrin saturation ($p < 0.05$) which paralleled an increase in serum iron concentration levels in both the groups. This significant improvement is contrary to what was observed amongst cancer patients with anaemia of chronic disease who were given 200 mg of lactoferrin along with erythropoietin.[84] Hence the beneficial effect of lactoferrin that is seen in cases of nutritional iron deficiency does not extrapolate to Anaemia of Chronic Disease.

There was no significant difference in the iron parameters and haemoglobin levels from base line to post-therapy between Groups II and III thereby quelling any belief on the possible haematological benefits of combining ferrous sulphate and lactoferrin fortified bovine colostrum.

The participants who received lactoferrin fortified bovine colostrum reported fewer adverse effects (4%, 13%) than those on ferrous sulphate (36%). This is in accordance with previous studies that have also noted considerable adverse effects with ferrous sulphate.[85] The most common adverse effect reported in this study was gastritis, followed by altered bowel movements. Further studies are required to

determine the role of lactoferrin fortified bovine colostrum in reducing the incidence of adverse drug reactions when given in combination with ferrous sulphate as has been demonstrated by the lower incidence of adverse effects reported in the arm that received both.

Significant improvement in haemoglobin and iron parameters were observed in participants who received lactoferrin fortified bovine colostrum either as a single agent or in combination with ferrous sulphate.

The mechanism by which lactoferrin improves iron parameters and hematologic parameters is yet to be understood. The iron binding capacity of lactoferrin, the iron content of lactoferrin fortified bovine colostrum or the IL-6 mediated hepcidin regulation may contribute to the effect of lactoferrin in iron metabolism.

A lower incidence of adverse drug reactions amongst participants on lactoferrin fortified bovine colostrum further justifies the utility of lactoferrin fortified bovine colostrum in correcting iron deficiency anaemia.

A study of larger scale may be carried out to further ascertain the efficacy of lactoferrin fortified bovine colostrum in treating iron deficiency. Further studies with purified lactoferrin are warranted to avoid difficulties in interpretation of the data. A study duration of at least 3 months or more would be desirable to assess the effect of lactoferrin on iron stores. Zinc protoporphyrin assay and RBC indices and counts would provide a better assessment of iron status of an individual.

CONCLUSION

Iron deficiency affects more than 2 billion people globally, with greater prevalence noted amongst women and children. When left untreated, it is associated with easy fatigability, decreased work performance, poor pregnancy outcomes and delayed developmental milestones. Hence it is imperative to rectify the iron deficiency in an individual. Oral ferrous sulphate, the most commonly prescribed drug for treating this condition, is associated with 25 to 40% incidence of adverse effects. This along with its variable bioavailability emphasise a need for better oral formulations. Lactoferrin, a glycoprotein structurally resembling transferrin, is believed to play a role in iron absorption. Previous studies conducted in Italian pregnant women and Japanese athletes have shown beneficial effects of bovine lactoferrin in treating iron deficiency anaemia.

Our study results demonstrate significant improvement in iron parameters and haemoglobin levels in those on lactoferrin fortified bovine colostrum compared to oral ferrous sulphate. The participants on lactoferrin fortified bovine colostrum reported fewer adverse effects and adhered to their treatment regimen more faithfully compared to those on the ferrous sulphate regimen. No improvement in iron and haematological parameters was noted on addition of ferrous sulphate to lactoferrin fortified bovine colostrum though the combination demonstrated lower incidence of adverse drug reactions.

Hence lactoferrin fortified bovine colostrum is more efficacious is treating iron deficiency anaemia compared to oral ferrous sulphate and is associated with fewer adverse drug reactions and greater compliance making it a suitable alternative to the current treatments available.

BIBLIOGRAPHY

1. Shersten Killip, John M. Bennett, Mara D. Chambers. Iron Deficiency Anaemia. American Family Physician. March 1, 2007; Vol. 75, No 5: 671 – 678.

2. Ministry of Health and Family Welfare Government of India. Guidelines for Control of Iron Deficiency Anaemia. 2013; Table 2.1:6.

3. Manu Tiwari, Col. Jyoti Kotwal , Anupam Kotwal, Maj Priyanka Mishra, Brig Vibha Dutta, Brig Sanjiv Chopra. Correlation of haemoglobin and red cell indices with serum ferritin in Indian women in second and third trimester of pregnancy. Medical Journal of Armed Forces India. 2013; 69: 31-36.

4. Matthew W. Short, Jason R., Domagalski. Iron Deficiency Anemia: Evaluation and Management. American Family Physician. Jan 2013; 87(2): 98-104.

5. L. Adlerova, A. Bartoskova, M. Faldyna Lactoferrin: A review Veterinarni Medicina. 2008; 53(9): 457–468.

6. Alex D. Sheftel, Anne B. Mason, Prem Ponka. The Long History of Iron in the Universe and in Health and Disease. Biochimica et Biophysica Acta. Mar 2012;1820(3): 161–187.

7. Guerinot M.L., Microbial iron transport. Annual Review of Microbiology. 1994; 48:743–72.

8. De Luca N.G., Wood P.M., Iron uptake by fungi: contrasted mechanisms with internal or external reduction. Advances in Microbial Physiology. 2000;43:39-74.

9. Martin Hynes. Iron Metabolism. Journal of Clinical Pathology.1948;1:57.

10. S.S. Nadadur, K. Srirama and Anuradha Mudipalli. Iron transport & homeostasis mechanisms: Their role in health & disease. The Indian Journal of Medical Research. October 2008;128:533-544.

11. Karen H. C. Lim, Lynn J. Riddell, Caryl A. Nowson, Alison O. Booth, and Ewa A. Szymlek-Gay. Iron and Zinc Nutrition in the Economically-Developed World: A Review. Nutrients. Aug 2013; 5(8): 3184–3211.

12. Namik Özbek. Concise Review: Absorption And Transport of Iron. Medical Journal of Islamic World Academy of Sciences. 2010;18(4):133-138,

13. Adriana Donovan, Cindy N.Roy, and Nancy C. Andrews. The Ins and Outs of Iron Homeostasis. Physiology. 2006; 21:115–123.

14. F. Dupic, S. Fruchon, M. Bensaid, O. Loreal, P. Brissot, N. Borot, M. P. Roth and H. Coppin. Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. Gut. Nov 2002; 51(5): 648–653.

15. Majid Shayeghi, Gladys O., Latunde-Dada,Jonathan S. Oakhill, Abas H. Laftah, Ken Takeuchi et al. Identification of an Intestinal Heme Transporter. Cell. September 9, 2005; Vol.122:789–801.

16. Tenhunen R., Marver H.S., Schmid R., The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proceedings of the National Academy of Sciences U S A. 1968;61:748–755.

17. Elizabeth L. Mackenzie, Kenta Iwasaki, Yoshiaki Tsuji. Intracellular Iron Transport and Storage: From Molecular Mechanisms to Health Implications. Antioxidants and Redox Signalling. 2008; 10(6):997–1030.

18. P.S. Oates. The role of hepcidin and ferroportin in iron absorption. Histology Histopathology. 2007; 22:791-804.

19. Luigi Messori and Felix Kratz. Transferrin: From Inorganic Biochemistry to Medicine Metal-Based Drugs. 1994; 1(2-3):161–167.

20. Andrea U. Steinbicker, Martina U. Muckenthaler. Out of Balance—Systemic Iron Homeostasis in Iron-Related Disorders. Nutrients. 2013; 5:3034-3061.

21. Pauline Lee, Hongfan Peng, Terri Gelbart, Lei Wang, and Ernest Beutler. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proceedings of the National Academy of Sciences. February 8, 2005; 102(6): 1906 –1910.

22. Nazanin Abbaspour, Richard Hurrell, and Roya Kelishadi Review on iron and its importance for human health. Journal of Research in Medical Sciences. Feb 2014;19(2): 164–174.

23. Kanjaksha Ghosh. Non haematological effects of iron deficiency - A perspective. Indian Journal of Medical Sciences. 2006;60:30-37.

24. John L. Beard. Iron Biology in Immune Function, Muscle Metabolism and Neuronal Functioning. Journal of Nutrition. 2001 Feb;131(25-2):568-579.

25. WHO Global Database on Anaemia, Geneva, World Health Organization. 2008.

26. WHO. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization. 2011.

27. Alka Kriplani, Reeta Mahey, Biswa Bhusan Dash, Vidushi Kulshreshta, Nutan Agarwal and Neerja Bhatla Intravenous iron sucrose therapy for moderate to severe anaemia in pregnancy. Indian Journal of Medical Research. July 2013; 138: 78-82.

28. Carole Warnes, Michael Honey, Nicholas Brooks, John Davies, Angela Gorman, Norman Parker. Mechanical haemolytic anaemia after valve repair operations for non-rheumatic mitral regurgitation. British Heart Journal 1980; 44: 381-385.

29. Charles Parker, Mitsuhiro Omine, Stephen Richards, Jun-ichi Nishimura, Monica Bessler, Russell Ware, Peter Hillmen, Lucio Luzzatto, Neal Young, Taroh Kinoshita, Wendell Rosse, Gerard Socié. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. December 1, 2005;106 (12): 3699–3709.

30. Achille Iolascon, Luigia De Falco, and Carole Beaumont. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. Haematological. 2009; 94(3):395-408.

31. Richard Hurrell and Ines Egli. Iron bioavailability and dietary reference values. American Journal of Clinical Nutrition. May 2010; 91(5): 1461-1467.

32. Terri D. Johnson-Wimbley and David Y. Graham. Diagnosis and management of iron deficiency anemia in the 21st century. Therapeutic Advances in Gastroenteroly. 2011;4(3):177-184

33. Elnicki D.M., Shockcor W.T., Brick J.E., Beynon D.Evaluating the complaint of fatigue in primary care: diagnoses and outcomes. American Journal of Medicine. Sep 1992; 93(3):303-306.

34. Lena Hulthe´n. Iron deficiency and cognition. Scandinavian Journal of Nutrition 2003; 47(3):152-156.

35. Lozoff B., Jimenez E., Wolf A.W. Long-term developmental outcome of infants with iron deficiency. New England Journal of Medicine. 1991 Sep 5;325(10):687-694.

36. Joint World Health Organization/Centers for Disease Control and Prevention Technical Consultation on the Assessment of Iron Status at the Population Level, Geneva, Switzerland, 6–8 April 2004.

37. World Health Organization /UNICEF/UNU. Iron Deficiency Anaemia: Assessment, Prevention, and Control. A Guide for Programme Managers. Geneva, Switzerland: World Health Organization; 2001.

38. Ami Ballin, Michael Berar,Uri Rubinstein, Yesheayahu Kleter, Ariela Hershkovitz, Dina Meytes, Iron State in Female Adolescents. American Journal of Diseases of Children. 1992;146(7):803-805.

39. Vivek kumar, Sourabh Aggarrwal, Alka Sharma, Vishal Sharma Nailing the diagnosis : Koilonychia The Permenente Journal 2012; 16(3):65.

40. Haas J., Rahn M., Venkatramanan S., Marquis G.S., Wenger M.J., Murray-Kolb L.E., Wesley A.S., Reinhart G.A.Double-fortified salt is efficacious in improving indicators of iron deficiency in female Indian tea pickers. Journal of Nutrition. Jun 2014;144(6):957-64.

41. Mann S.K., Kaur S., Bains K. Iron and energy supplementation improves the physical work capacity of female college students. Food and Nutrition Bulletin. Mar 2002 ;23(1):57-64.

42. Alice M. Nyakeriga,Marita Troye-Blomberg, Jeffrey R. Dorfman, Neal D. Alexander, Rune Back, Moses Kortok, Alex K. Chemtai, Kevin Marsh, and Thomas N. Williams. Iron Deficiency and Malaria among Children Living on the Coast of Kenya. The Journal of Infectious Diseases. 2004;190(3): 439-44.

43. K. Kalaivani. Prevalence & consequences of anaemia in pregnancy. Indian Journal of Medical Research. November 2009; 130: 627-633.

44. Soewondo S, Husaini M, Pollitt E. Effects of iron deficiency on attention and learning processes in preschool children: Bandung, Indonesia. American Journal of Clinical Nutrition. Sep 1989; 50(3):667-673.

45. Keith J. Collard. Review Article: Iron Homeostasis in the Neonate. Pediatrics April 2009; 123(4):1208-1216.

46. Freddy J. Troost, Jan Steijns, Wim H. M. Saris and Robert-Jan M. Brummer. Gastric Digestion of Bovine Lactoferrin in vivo in Adults. Journal of Nutrition. August 1, 2001; 131(8): 2101-2104.

47. Jay B. Wish. Assessing Iron Status: Beyond Serum Ferritin and Transferrin Saturation. The Clinical Journal of the American Society of Nephrology. September 2006; 1(1): 54-58.

48. Muriel Wollmann, Branca Maria Cerezer Gerzsonb, Vanessa Schwertc, Rafael Weber Figuerad, Guilherme de Oliveira Ritzeld. Reticulocyte maturity indices in iron deficiency anemia. Revista Brasileira de Hematologia e Hemoterapia. January–February /2014, 36(1): 25–28.

49. Kenneth Kaushansky, Marshall A. Lichtman, Beutler E., Thomas J.Kipps, Josef Prchal, Uri Seligsohn.Disorders of Iron Metabolism.Williams Hematology.8th edition (e-version). McGraw-Hill; 2010.

50. Myfanwy J. Borel, Scot M. Smith, Janice Der, and John L. Beard Day-to-day variation in iron-status indices in healthy men and women. American Journal of clinical Nutrition. l99l;54:729-735.

51. J.V. Gnanou, S. Muthayya and A.V. Kurpad. Biological Variation of Plasma Ferritin in Healthy Adult Males in South Indian Population – A Sample Study. Indian Journal of Clinical Biochemistry. 2006;21 (1) 193-195.

52. Hachiro Yamanishi,Shigeru Iyama,Yoshihisa Yamaguchi,Yuzuru Kanakura and Yoshinori Iwatani.Total Iron-binding Capacity Calculated from Serum Transferrin Concentration or Serum Iron Concentration and Unsaturated Iron-binding Capacity. Clinical Chemistry. January 2003; 49:1 175-178.

53. Labbé RF, Dewanji A. Iron assessment tests: transferrin receptor vis-à-vis zinc protoporphyrin. Clinical Biochemistry. Mar 2004 ;37(3):165-74.

54. Fernando Bermejo and Santiago García-López. A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases. World J Gastroenterol. Oct 7, 2009; 15(37): 4638–4643.

55. Kenneth Kaushansky; Thomas J. Kipps. Ln: Laurenc Brunton, Bruce Chabner, Bjorn Knollman Editors. Goodman & Gilman's the Pharmacological Basis of Therapeutics. Hematopoietic Agents: Growth Factors, Minerals, and Vitamins. 12e. McGraw-Hill Professional:1076-1085.

56. M Harrington, C. Hotz, C. Zeder, G. O. Polvo, S. Villalpando, M. B. Zimmermann, T. Walczyk, J. A. Rivera and R. F. Hurrell. A comparison of the bioavailability of ferrous fumarate and ferrous sulfate in non-anemic Mexican women and children consuming a sweetened maize and milk drink. European Journal of Clinical Nutrition. 2011; 65:20–25.

57. Dora I.A. Pereira, Susana S. Couto Irving, Miranda C.E. Lomer and Jonathan J. Powell. A rapid, simple questionnaire to assess gastrointestinal symptoms after oral ferrous sulphate supplementation. BMC Gastroenterology. 2014; 14:103.

58. Scott B. Silverstein and George M. Rodgers. Parenteral Iron Therapy Options. American Journal of Hematology. 2004;76:74–78.

59. Robert Provenzano, Brigitte Schiller, Madhumathi Rao, Daniel Coyne, Louis Brenner and Brian J.G. Pereira. Ferumoxytol as an Intravenous Iron Replacement Therapy in Hemodialysis Patients. Clinical Journal of the American Society of Nephrology. Feb 2009; 4(2): 386–393.

60. Jeremy Wally and Susan K. Buchanan. A structural comparison of human serum transferrin and human lactoferrin. Biometals. Jun 2007; 20(3-4): 249–262.

61. Sun, X.L., Baker, H.M., Shewry, S.C., Jameson, G.B., Baker, E.N. Structure of recombinant human lactoferrin expressed in Aspergillus awamori. Journal: Acta Crystallographica 1999;55: 403-407.

62. Artym J. [The role of lactoferrin in the iron metabolism. Part I. Effect of lactofferin on intake, transport and iron storage] Postępy Higieny i Medycyny Doświadczalnej (Online). Nov2008; 3;62:599-612.

63. Lourdes Sanchez, Miguel Calvo, Jeremy H. Brock. Biological Role of Lactoferrin. Archives of Disease in Childhood 1992; 67:657-661

64. Peter Ferenc Levay, Margaretha Viljoen Lactoferrin: A General Review Haematologica. 1995; 80:252-267

65. Suzuki Y.A., Shin K., Lönnerdal B. Molecular cloning and functional expression of a human intestinal lactoferrin receptor Biochemistry. Dec 2001 ;40(51):15771-9.

66. Kouichirou Shin, Hiroyuki Wakabayashi, Koji Yamauchi, Tomoko Yaeshima, and Keiji Iwatsuki Recombinant Human Intelectin Binds Bovine Lactoferrin and Its Peptides Biological and Pharmaceutical Bulletin. 2008; 31(8) 1605—1608.

67. Cox T.M., Mazurier J., Spik G., Montreuil J., Peters T.J. Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine. Biochimica et Biophysica Acta. Nov 1979 ;588(1):120-128.

68. Pauline P. Ward, Marisela Mendoza-Meneses, Grainne A. Cunningham, and Orla M. Conneely. Iron Status in Mice Carrying a Targeted Disruption of Lactoferrin. Molecular and Cellular Biology. Jan 2003; 23(1): 178–185.

69. Kawakami H., Makiko Hiratsuka and Dosako S., Effects of Iron-saturated Lactoferrin on Iron Absorption. Agricultural and Biological Chemistry, 1988;52 (4), 903-908.

70. Gifford J.L., Hunter H.N., Vogel H.J. Lactoferricin: A lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cellular and Molecular Life Sciences. Nov 2005; 62(22):2588-2598.

71. Barry-Lee Waarts, Onwuchekwa J.C. Anekea, Jolanda M. Smit, Koji Kimatac, Robert Bittmand, Dirk K.F. Meijerb,Jan Wilschut. Antiviral activity of human lactoferrin: Inhibition of alphavirus interaction with heparan sulphate. Virology. 2005;333:284–292.

72. Tetsuya Tanaka, Yoshitaka Omata, Tsuya Isamida, Atsushi Saito, Keiichi Shimazaki, Koji Yamauchi et al. Growth Inhibitory Effect of Bovine Lactoferrin to Toxoplasma gondii Tachyzoites in Murine Macrophages: Tyrosine Phosphorylation in Murine Macrophages Induced by Bovine Lactoferrin. Journal of Veterinary Medical Science. 1998; 60(3):369-371.

73. Michal Machnicki, Michal Zimecki, and Tadeusz Zagulsklt. Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo International Journal of Experimental Pathology. 1993;74:433-439.

74. Eve Damiens, Joël Mazurier, Ikram El Yazidi, Maryse Masson, Isabelle Duthille, Geneviève Spik, Yolande Boilly-Marer Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells Biochimica et Biophysica Acta. April 1998; 1402(3)24: 277–287

75. Sawatzki G, Rich I.N. Lactoferrin stimulates colony stimulating factor production in vitro and in vivo. Blood Cells. 1989;15(2):371-85.

76. B.R.Thapa. Health Factors in Colostrum.Indian Journal of Pediatrics. July 2005; 72:579-581.

77. Rosalba Paesano, Francesca Berlutti,Miriam Pietropaoli,Fabrizio Pantanella,Enrica Pacifici ,William Goolsbee , Piera Valenti. Lactoferrin efficacy versus ferrous sulfate in curing iron deficiency and iron deficiency anemia in pregnant women. Biometals. 2010; 23:411–417.

78. Rosalba Paesano, Francesca Berlutti,Francesca Torcia,Enrica Pacifici, Valeria Ebano,Massimo Moscarini, Piera Valenti. Oral Administration of lactoferrin increases haemoglobin and total serum iron in pregnant women. Biochem. Cell Biology. 2006; 84:377-380.

79. Mehta B C. Iron deficiency amongst nursing students. Indian Journal of Medical Sciences. 2004;58:389-393.

80. Chandrakala Kambar, Zahedabano, Meenakumari A. Comparative study of efficacy and safety of Iron polymaltose complex with ferrous sulphate in antenatal women with moderate anemia. International Organization of Scientific Research Journal of Dental and Medical Sciences. Jul – Aug 2013; 9(1):09-13.

81. John W. Adamson. Ln: Longo, Fauci, Kasper, Hauser, jameson, Loscalzo. Editors. Harrison's Principle of Internal Medicine. Iron Deficiency and Other Hypoproliferative Anemias. 17th edition. McGraw-Hill Professional: 628-633.

82. Natsue Koikaway, Isao Nagaoka,Masahiro Yamaguchi,Hirokazu Hamano, Koji Yamauchi,Keisuke Sawaki. Preventive Effect of Lactoferrin Intake on Anemia in Female Long Distance Runners.Biosci. Biotechnol. Biochem. 2008;72 4): 931–935.

83. Carmine Nappi, Giovanni Antonio Tommaselli, Ilaria Morra, Mariangela Massaro, Carmen Formisano and Costantino Di Carlo.Efficacy and tolerability of oral bovine lactoferrin compared to ferrous sulfate in pregnant women with iron deficiency anemia: A prospective controlled randomized study. Acta Obstetricia et Gynecologica. 2009; 88: 1031-1035.

84. Antonia Maccio, Clelia Madeddu et al. Efficacy and Safety of Oral Lactoferrin Supplementation in Combination with rHuEPO- for the Treatment of Anemia in Advanced Cancer Patients Undergoing Chemotherapy: Open-Label,Randomized Controlled Study The Oncologist 2010;15:894-902.

85. Ariani Impieri de SouzaI; Malaquias Batista FilhoI; Cristiane Campello BresaniI; Luiz Oscar Cardoso FerreiraII; José Natal Figueiroa Adherence and side effects of three ferrous sulfate treatment regimens on anemic pregnant women in clinical trial Cad. Saúde Pública. June 2009; 25(6): 1225-1233.

86. Jennifer K. Chow, Barbara G. Werner, Robin Ruthazer, David R. Snydman. Increased Serum Iron Levels and Infectious Complications after Liver Transplantation. Clinical Infectious Diseases. 2010;51 (3):16-23.

87. Jürgen Stein, Franz Hartmann & Axel U. Dignass.Diagnosis and management of iron deficiency anemia in patients with IBD. Nature Reviews Gastroenterology and Hepatology. November 2010;7:599-610.

Patient Information Sheet

Anaemia is a disease that presents with easy fatigability, lack of vitality, weakness, faintness, palpitations, headache and breathlessness, palpitations.

It is caused by reduced amount of haemoglobin in red blood cells which are instrumental in delivering oxygen to your tissues.

Laktrum is bovine colostrum fortified with lactoferrin. Laktrum is a natural nutritional supplement providing rich nutrients that promote immunity, growth and good health. Colostrum is found in first 24-36 hrs of mother's milk and is rich in vitamins and minerals.

Lactoferrin in bovine colostrum will increase the absorption of iron from your body and hence result in increased synthesis of haemoglobin. This will result in better oxygen delivery to your tissues and make you feel less tired, and more active.

Laktrum does not contain any substances that can induce allergy and is safe for consumption.

Take 2g (one spoonful) of laktrum and mix it in one cup water or milk. This is to be taken at bedtime for a period of 30 days.

PATIENT CONSENT FORM

Study Title : Comparative Study of Efficacy of Lactoferrin fortified bovine colostrum with Oral Iron in the Treatment of Iron Deficiency Anaemia

Study Center: Kilpauk Medical College and Hospital, Chennai-10

Patient Name : O.P. No.:

Patient Age/sex:

I confirm that I have understood the purpose and procedure of the above study. I had the opportunity to ask questions and all my doubts have been answered satisfactorily.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time without my legal rights being affected.

I understand that the sponsor of the clinical study, members of the ethical committee and the investigators involved in the study will not need my permission to look at my health records, both in respect to the current study and any other further research that may be conducted in relation to it. However, I understand that my identity will not be revealed in any information released to third parties or published, unless as required under the law. I agree not to restrict the use of any data or results that may arise from this study.

I hereby consent to participate in this study.

I hereby give permission to undergo complete clinical examination and diagnostic tests including withdrawal of 8ml of blood at the beginning and end of the study.

Patient Signature/ Thumb Impression :

Patient Name and address :

Witness Signature/ Thumb Impression :

Witness Name and address :

Investigator's Signature :

Name of the Investigator :

Place:

Date:

COMPARATIVE STUDY OF EFFICACY OF LACTOFERRIN FORTIFIED BOVINE COLOSTRUM WITH ORAL IRON IN THE TREATMENT OF IRON DEFICIENCY ANAEMIA

CASE REPORT FORM

Name	:	D/O:
Age	:	
Sex	:	
Address	:	
Ref No.	:	
History	:	
Past H/o	:	
H/o of bleeds	:	
H/O of Renal or Liver disease	:	
H/o of Blood transfusion	:	
H/o of medication	:	
Past Medical H/o	:	

Signs

General: Pallor: Icterus: Clubbing: Cyanosis: Edema:

Vitals: BP: PR: Temp:

CVS :

R.S :

P/A :

CNS :

Investigations

Test	Baseline	Post- Therapy
Peripheral Smear		
Hb%		
Sr. Iron		
Sr. Ferritin		
Transferrin Saturation		
Total Iron binding capacity		
TC		
DC		
ESR		
SGOT		
SGPT		
Bilirubin		
Urea		

Creatinine		

தொலைபேசி தகவல் தாள்

இந்த சோலார்மற்று மரினாக கணம்பாடகுல் பலவிதம் தலைவர்கள்,படப்பு தலைவரி மற்றும் ரூச்சாடப்பு போன்ற தன்மைகளை உடையது.

இந்த சிலம்புகளில் உள்ள ஹரிமொழிகளில் அலுவலகமும் இந்தோர் ஏற்படுகிறது. இது உங்கள் நிகழ்வுக்கு ஆசாரிகள் வழங்கும் கருவியாக பயன்படுகிறது.

வேட்ரம் என்பது வேர்டெரபெரின் கொண்ட ஒருவகையான சிப்பாய் கொலம்ப்ரம் வேட்ரம் தோல் எதிர்ப்பு சக்தி வளர்ச்சி மற்றும் நல்ல ஆரோக்கியத்தை மேம்படுத்தும் மற்றும் அதிலை ஊட்டச்சத்து வழங்கும் ஒரு இயற்கை ஊட்ட சத்து.

கொலம்ப்ரம் என்பது தாய் பாலில் முதல் 24-36 மணிக்குள் காணப்படும் கைப்பிடிகள் மற்றும் தாதுக்கள் நிறைந்து உள்ளது.

வேர்டெரபெரின் நல்ல வளர்ச்சி மற்றும் குணமடைவதற்கும் முன்னேற்பாக இருக்கிறது சிப்பாய் கொலம்ப்ரம் உள்ள வேர்டெரபெரின் உங்கள் உடலில் இருந்து இரும்புச்சத்து உறிஞ்சப்படுவதை அதிகரிக்கும் அதன் விளைவாக ஹரிமொழிகளில் எண்ணிக்கை அதிகரிக்கும் இதன் மூலமாக உங்கள் நிகழ்வுக்கு அதிக ஆசாரிகள் கிடைப்பதால் நீங்கள் கொற்கைபாலம் மற்றும் அதை சுற்றுப்புறம் இருப்பீர்கள்.

வேட்ரமில் ஒவ்வாமை தூண்டக் கூடிய எந்த பொருட்களும் இல்லை மற்றும் உட்கொள்ள பாதுகாப்பானது.

இரவு உறங்குவதற்கு முன் அதிரம் வேட்ரம் ஒரு வளர்ச்சித்து ஒரு கப் தண்ணீர் அல்லது பால் கலந்து 30 நாட்களுக்கு எடுக்கப்பட வேண்டும்.

தொலைபேசி ஒப்புதல் படிவம்

ஆய்வு தலைப்பு: இருப்புச்சத்து குறைபாடு இரத்தசோகையின் சிகிச்சையில் வாய்வழி அயர்ன் மற்றும் வேர்டெரபெரின் செறிவூட்டப்பட்ட சிப்பாய் கொலம்ப்ரமின் திறனை ஒப்பிடுதல் ஆய்வு

ஆய்வு மையம்: கிழ்ப்பாக்கம் மருத்துவ கல்லூரி, சென்னை - 10

தொலைபேசியின் பெயர்:

முதி எண்:

தொலைபேசியின் வயது / பாலினம்:

இந்த ஆய்வின் நோக்கம் மற்றும் நடைமுறை தெளிவாக புரிந்தது என்று உறுதி அளிக்கிறேன். நான் கேள்விகள் கேட்க வாய்ப்பு கிடைத்தது மற்றும் அவைத்து என் சந்தேகம் திருப்திகரமாக பதிலளிக்கப்பட்டுள்ளது.

இந்த ஆய்வில் என் பங்கு தன்னார்வலருடையது என்றும் மற்றும் என் சட்ட உரிமைகள் பாதிக்கப்படாமல் எந்த நேரத்திலும் நான் ஆய்வில்இருந்து வெளியேறலாம் என்றும் அறிந்திருக்கிறேன்.

மருத்துவ ஆய்வு ஆதாயமானது தெரிவுசெய்த குழு மற்றும் ஆய்வு சம்பந்தப்பட்ட விசாரணை உறுப்பினர்கள்தற்போது நடக்கும் ஆய்வு மற்றும் இது தொடர்பாக எதிர்காலத்தில் நடத்தப்படும் எந்த ஆய்வினும் என் அனுமதியினறி என் மருத்துவ பதிவுகளை பார்க்கலாம் என்றும் எனது

INSTITUTIONAL ETHICAL COMMITTEE
GOVT.KILPAUK MEDICAL COLLEGE,
CHENNAI-10
Ref.No.2318/ME-1/Ethics/2012 Dt:04.04.2013
CERTIFICATE OF APPROVAL

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "A Comparative Study of efficacy of Lactoferrin fortified bovine colostrums with iron in the Treatment of Iron Deficiency" - For IEC Approval submitted by Dr.R.Tarumi, MD (Pharm), PG Student, KMC, Chennai-10.

The Proposal is APPROVED.

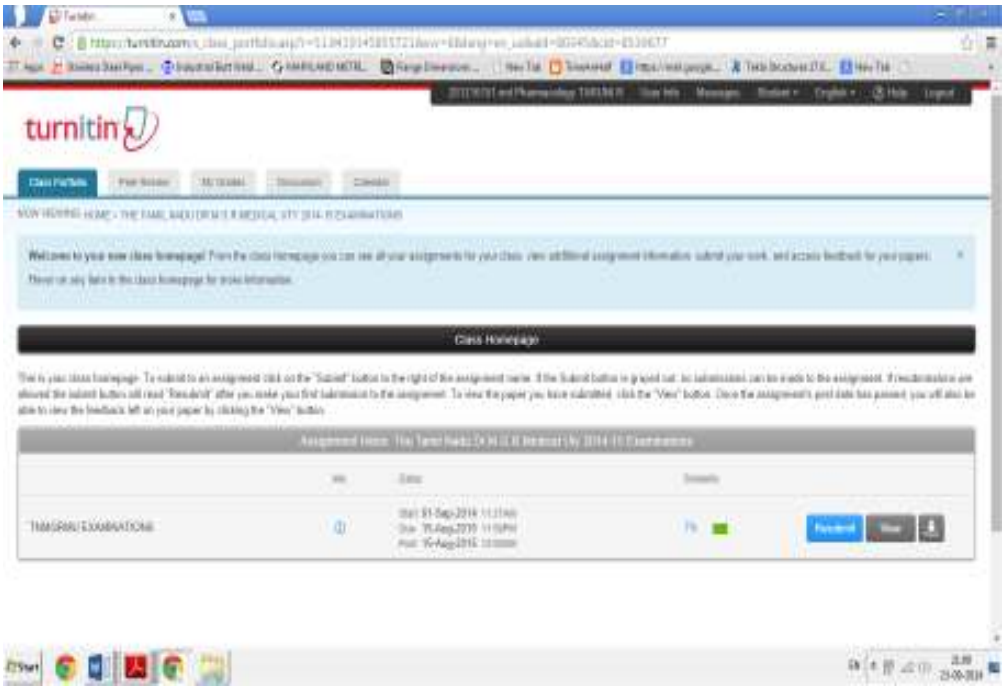
The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

GOVT. KILPAUK MEDICAL COLLEGE
29 MAY 2013
Kilpauk, Chennai-10.

29/5/13
CHAIRMAN,
Ethical Committee
Govt.Kilpauk Medical College.Chennai

PLAGIARISM

CERTIFICATE



Group	Name	Age(years)	Hb		Sr. Iron		Sr. Ferritin		TIBC		UIBC		TSAT	
			BL (g/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)
II	Sugunya	19	8	8	57.9	55.92	23.94	28.32	372.99	360.3	315.09	304.38	15.522209438	15.5204
II	Sangeeswari	19	8	9.2	56.19	161.05	0.666	2.84	681.67	318.51	625.68	157.46	8.244957516	90.56356
II	Kalaiselvi	19	9.9	10.8	78.29	97.44	14.93	16.5	482.67	242.47	404.38	244.03	16.228469183	28.38925
II	Muthulakshmi	20	8	11.8	19.76	25.16	4.3203	5.44	498	342.16	478.26	317.02	5.963855422	7.352055
II	Karpagavigneswari	19	9	9.4	46.13	69.81	2.434	7.965	502.07	327.02	448.54	266.61	11.8256751	18.57268
II	Lakshmi	19	9.5	11	63.17	55.69	7.7455	7.4225	498	386.22	454.83	330.53	8.660674699	14.41924
II	R.D.Sonia	18	9.3	9.8	39.6	36.94	3.229	4.321	532.26	341.62	512.66	324.75	3.682410852	4.958
II	Suguthakari	19	9	10.6	62.76	82.82	3.996	6.721	477.84	326.7	415.08	243.88	13.13410347	25.35047
II	S.Sathya	19	9.6	10	80.66	83.16	6.68	3.362	535.81	379.08	445.15	295.92	15.30034188	21.93737
II	Lavanya	19	9.08	10	31.84	34.71	44.11	42.6	516.93	441.78	485.09	407.07	6.159661317	7.856852
II	Kaviya	19	8.9	8.8	49.74	25.26	8.93	6.957	402.03	387.27	352.29	362.01	12.37221193	6.522084
II	Divya	19	8	9	48.03	48.15	13.83	11.47	514.92	325.65	466.89	277.53	9.32706255	14.77967
II	Jaya Priya	19	10	10.4	29.61	46.26	9.246	5.023	505.65	476.34	476.04	430.08	5.853829131	9.731551
II	D.JeganPriya	19	10	12.8	23.03	25.68	46.675	28.79	463.71	482.6	440.68	376.92	4.966966611	6.378336
II	Kala	18	9.5	9.8	53.55	94.2	25.355	6.308	437.1	302.49	383.55	208.29	12.2912011	31.14133
II	P.Gurulakshmi	19	10	11	83.39	80.77	34.15	14.93	654.45	621.39	591.06	540.62	9.685995874	12.99828
II	Ilakkiya	21	11	11.3	76.56	102.13	80.55	18.16	514.31	398.92	437.55	296.67	13.801754468	25.60746
II	Rita	19	11	12	47.35	46.43	126.1	153.37	462.09	491.13	414.98	444.7	10.99698366	9.453709
III	Baby	19	9.6	10.4	27.3	40.02	8.3	6.177	333.07	431.82	305.57	391.8	8.198797156	9.26775
III	Esther	19	8.6	10.2	40.92	87.69	91.65	191.5	451.2	287.52	410.28	199.83	9.066914956	30.48185
III	Kavitha	19	10	10.3	52.11	86.09	15.56	38.16	474.6	358.08	422.49	271.36	10.97977244	24.21157
III	M.Unathilaheswari	19	8	10.4	26.71	112.6	5.325	5.533	536.49	338.46	509.78	225.86	4.978657571	33.26813
III	Nasariya*	20	8	11.4	37.24	29.21	9.829	8.017	686.3	314.94	649.06	285.73	1.423437717	9.274782
III	Nagasundari	20	8.2	8.1	75.85	75.46	59.38	39.67	524.19	397.83	448.66	234.17	14.40889764	23.92876
III	Muragathumani	20	10	10.8	66.92	76.86	75.21	73.948	503.64	305.92	457.12	429.06	13.20784685	15.19213
III	Vijayalakshmi V.	19	8	8	10.79	14.86	6.9605	5.852	521.37	457.83	510.56	442.97	2.069543338	3.245346
III	Vanaka	19	8	9.4	21.84	30.25	1.803	5.077	542.76	469.26	520.92	438.98	4.023819867	6.466732
III	Viji	18	8	9.6	51.45	35.34	NA	NA	435.9	380.12	384.45	354.78	15.656380125	21.27889
III	Abhishek	19	10	10.2	14.6	43.42	12.69	11.95	416.13	375.51	401.53	332.09	3.508518972	12.09555
III	Banumathi	19	10	10.6	69.21	75.22	14.51	19.25	321.78	450.69	252.57	375.47	16.631802179	20.05142
III	T.Vasanthi	18	9.6	10	33.16	66.97	2.216	4.921	460.97	416.55	350.81	349.58	7.668975129	16.0773
III	S.Sachandradevi	19	9	9.2	63.29	72.35	12.6	7.9945	608.88	678.63	545.59	606.08	15.667500498	17.81688
III	S.Girbika Devi	19	11	11	28.42	17.29	3.299	8.388	595.8	415.32	567.38	377.93	4.770057064	9.082697
III	Sangeetha T.	19	9	11.5	92.9	84.85	37.859	5.534	528.63	260.07	256.12	175.21	13.52534408	28.42281

BL: Base line ; PT : Post Therapy

*** Reported Adverse Drug Reaction**

Group	Name	Age(years)	Hb		Sr. Iron		Sr. Ferritin		TIBC		UIBC		TSAT	
			BL (g/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)	BL(mcg/dl)	PT(g/dl)
I	Revathi	19	9.6	9	68.24	55.36	128.9	145.8	575.81	580.7	507.57	525.34	11.85113143	9.484324
I	Yamuna*	20	8	7.8	68.66	58.82	4.232	14.12	522.2	524.68	453.52	465.86	11.03825137	13.85043
I	Margret Asery	18	10	9.6	55.92	16.22	12.18	21.06	450.81	468.15	394.89	451.93	12.40432886	3.464701
I	Ayesha Jalini	21	10	11.2	37.65	35.26	8.78	16.26	577.44	847.81	539.81	613.79	6.519699373	5.744037
I	S.Mahalakshmi*	20	9.6	9.4	41.18	80.83	5.283	12.28	328.63	321.36	287.43	240.54	12.53869875	25.34937
I	P.Priyadharshini	19	10	10.4	29.41	49.67	17.04	22.65	457.65	515.73	428.04	466.06	6.470009835	9.631008
I	Sivajothi	20	10	9.8	71.3	75.22	4.058	8.405	534.69	535.08	457.19	459.86	14.49437992	14.057731
I	D.Ramani	19	10	10.6	45.4	74.67	10.96	14.98	585.48	541.14	540.08	466.47	7.756120241	13.79865
I	B.Ratnya*	19	10	10.2	18.68	72.77	7.82	11.3585	619.35	518.16	600.67	445.39	3.01608523	14.04392
I	K.Nanda Devi	18	10	9.9	64.08	80.67	5.391	6.231	451.2	585.9	387.12	505.43	14.20212766	13.75643
I	K.Manimeari	19	10	9.6	78.47	69.09	6.503	7.219	420.57	564.52	346.1	495.03	17.70492156	12.24739
I	S.Darlina	20	9.2	8.9	356.7	83.3	2.077	4.483	557.65	516.54	450.99	461.24	19.13386533	10.79585
I	S.EstherPrueda	21	10	9.9	46.22	16.53	0.399	2.655	996.37	997.58	887.13	581.05	9.919990886	2.766157
I	R.Geetha	19	8.2	8.4	46.54	60.33	18.86	19.75	545.58	545.97	499.04	505.64	8.530371346	7.384853
I	P.Maheeswari	18	11	11.4	78.37	70.2	4.019	4.792	597.99	552.84	519.62	482.64	13.10557035	12.69807
I	M.Sandhya	19	10.2	10	60.51	59	18.91	11.22	443.55	480.64	338.04	421.64	14.76647157	12.2755
I	N.Thiruvasagi	20	10	10.2	45.86	31.77	15.09	15.68	539.36	486.51	493.5	457.74	8.443009242	6.490164
I	Sumandevvi*	20	8.2	8	50.67	89.53	11.85	10.28	694.35	525.42	643.68	435.91	7.297472456	17.0359
I	Shiny J. *	19	10	9.9	19.47	12.59	9.926	4.792	527.52	420.96	508.03	408.37	3.690855323	2.990793
I	B.Kalavathi*	20	10.8	10.9	27.1	24.76	17.48	12.175	523.92	419.76	496.82	395	5.172545427	5.898609
I	B.Ramya*	18	10	10.2	8.82	13.1	36.705	11.56	505.26	444.75	496.44	431.65	1.74363391	2.945671
I	N.Ranjitha	19	10	10.1	32.57	64.24	20.9	5.113	530.25	439.92	477.88	375.68	9.871647536	14.60266
I	G.PadmaPriya*	19	10	10.4	55	62.5	28.655	14.4545	448.8	395.38	393.8	333.08	12.25498196	13.79454
I	K.Meena*	18	10.6	10.5	28.42	45.88	28.745	7.347	618.34	549.23	598.12	503.25	4.596698723	8.372625
I	A.Vasanthi*	18	11	10.8	64.87	64.73	51.895	13.742	554.43	483.86	489.56	417.33	11.70030482	13.43336
II	A.Vijayalakshmi	19	9.5	10	17.34	26.61	3.098	56.02	563.7	575.4	546.36	548.79	3.076104311	4.626609
II	V.Aiswar	19	9.6	10.4	51.32	164.74	16.55	58.58	649.2	417.21	617.68	252.47	4.624599261	39.48611
II	Kanchana	19	10	10.4	42.63	56.13	20.55	32.76	576.2	397.26	533.57	341.13	11.33173844	14.12929
II	Edwin Nirmala*	19	9.8	12.08	40.13	38.11	4.912	12.13	411.69	328.83	371.56	270.72	9.747625641	17.67175
II	Ruby	19	9.8	10.9	70.13	72.04	2.305	8.615	529.44	398.31	459.31	326.27	13.24607137	18.08602
II	K.Sheenaja Devi	20	10.08	10.8	41.32	33.38	6.179	11.14	558.06	339.9	516.74	306.52	7.404221768	10.49898
II	SivaKamari	19	10	10	62.63	113.62	1.91	6.466	625.41	378.3	562.78	264.68	10.01423066	30.03456

BL: Base line ; PT : Post Therapy
* Reported Adverse Drug Reaction

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